

ANNUAL REPORT  
OF  
PROGRAM ACTIVITIES  
NATIONAL INSTITUTE OF NEUROLOGICAL AND  
COMMUNICATIVE DISORDERS AND STROKE  
FISCAL YEAR 1981  
VOLUME II

U. S. DEPARTMENT OF HEALTH AND HUMAN SERVICES  
Public Health Service      National Institutes of Health















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NATIONAL INSTITUTE OF NEUROLOGICAL AND COMMUNICATIVE DISORDERS AND STROKE  
DC

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VOLUME II





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October 1, 1980 through September 30, 1981

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ANNUAL REPORT

October 1, 1980 through September 30, 1981

Office of the Director, Intramural Research Program

National Institute of Neurological and Communicative Disorders and Stroke

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Annual Report of the Scientific Director  
of the  
National Institute of Neurological and  
Communicative Disorders and Stroke

October 1, 1980 through September 30, 1981

The mission of the NINCDS Intramural Research Program (IRP) is to conduct neurosciences research through the direct operation of its laboratories and clinics. In facilities on the main NIH campus as well as at offsite locations in Rockville and Frederick, Maryland; Woods Hole, Massachusetts; and on the island of Guam, the IRP has continued to make substantial contributions to the explosive growth of neurosciences research. These vigorous efforts, ranging from basic neurobiologic probes to clinical trials of new therapeutic agents, promise to advance significantly our ability to prevent, ameliorate, or cure neurologic and communicative disorders. On the other hand, an unmistakable decline in support now threatens the timely realization of these important objectives.

The impressive scientific accomplishment of IRP investigators during the past fiscal year will be detailed in subsequent sections. This report summarizes administrative changes occurring during the past year as well as those planned for future implementation. Salient managerial factors challenging the immediate and long-term vitality of Program efforts are also reviewed.

The financial fortunes of the Program generally reflect those of the Institute. In recent years, it has been a matter of NINCDS policy to maintain the existing size of the IRP and to attempt to stabilize its funding. Since 1976 the Program has consistently received approximately 11-12% of the total NINCDS allocation. However, recent Congressional actions, instigated by NIH to protect the number of investigator-initiated research grants, inexplicably ignore the needs of Intramural scientists and threaten to destabilize their support. During fiscal year 1981 the total NINCDS allocation increased by 4.6%, but the proportion given IRP fell to only 9.5%. This decline resulted in a small reduction in the other objects budget, which when compounded by inflation, compelled cutbacks in IRP-controllable expenditures: Certain capital equipment purchases and scheduled laboratory renovations had to be deferred and several promising research initiatives were delayed. The ineluctable result has been a loss of Program efficiency and a diminished inability to exploit emerging research opportunities. While hardly severe in its immediate impact, the long-term implications of this trend, should it continue, will be substantial. Available budgetary predictions for FY 1982 hardly encourage an optimistic view of this situation.

Reductions in the allocation for IRP research and development contracts, anticipated as well as already realized, are a source of even more serious concern. By funding such activities as primate holding and reagent synthesis, these contracts provide essential support for high priority in-house investigations. In the past 5 years IRP contract obligations



averaged about \$3 million. In FY 1981 our contract budget fell to \$2.8 million, while at the same time the cost of the Program's existing contracts increased. For example, IRP's share of contractual costs for primate holding at the Frederick Cancer Research Center rose three-fold during the past two years, and now amounts to 15% of our entire research and development contract expenditure. Further reductions in IRP contract funds are anticipated next year, which will inevitably result in a diminution of the Program's scientific activity.

Previous annual reports have emphasized the increasing difficulties imposed by governmental personnel policies. The past year provided no relief from this frustrating situation. The gross inadequacies in the compensation offered senior IRP scientists, crowded at the Federal "pay cap", has not improved. Uncertainties concerning the Senior Scientific Service bonus system have been supplanted by wide-spread disillusionment over the fairness and size of these awards. Implementation of the Merit Pay system for GS grades 13 through 15 supervisors provided yet another source of contention and demoralization for monetary rewards that seemed relatively inconsequential. Extension of the merit pay system to all remaining civil service and wage board employees can be predicted to have its managerial benefits substantially exceeded by its administrative burdens. In all such matters, it seems incumbent upon NIH personnel management officials to seek tenable alternatives for policies which - however well intentioned and reasonable for other governmental agencies - stand in obvious conflict with NIH organizational needs and operating efficiency.

Various prohibitions on hiring throughout FY 1981 also challenged IRP management by compelling delays or even cancellation of some employment commitments and ultimately by reducing the size and altering the composition of the Program's work force. By June - July of 1981, the total number of IRP employees had fallen about 10% in comparison with the same months in 1980. This reduction has had a more deleterious impact than might be expected, since the overall loss in numbers disguises a 23% decline in key full-time permanent personnel, only partially offset by increases in the number of temporary full-time, intermittent, and special expert employees. The effects of the newly initiated full-time equivalent personnel accounting system are yet to be recognized; limitations on hiring continue to be rate limiting and no allocations of full-time equivalent hours to the IRP has been made.

Although the foregoing personnel limitations complicate the realization of IRP EEO objectives, nevertheless, significant strides continue to be made, particularly in the administrative area, in employment and advancement opportunities for minorities and women. IRP also continues to lead in the employment under the EEO Summer Employment Program of high school, undergraduate, and graduate students in the laboratories, branches, and the Scientific Director's Office. This program has provided on-the-job training and experience designed to encourage talented students to pursue biomedical research careers in the neurosciences.

Space available for IRP operations remained essentially unchanged during the past fiscal year. The Program presently occupies approximately 128,000 square feet of space (86,000 square feet in five on-campus buildings plus 32,000 square feet in three other locations). An additional 6,000 square

feet should become available for Program use when the Ambulatory Care Research Facility opens in FY 1982. This increment, with its associated opportunity for geographic consolidation, will markedly improve Program operating efficiency and convenience. Plans to upgrade and enlarge Building 9 also began this year. At present the NINCDS occupies approximately 40% of this facility. As new laboratory construction on the NIH reservation is considered unlikely in the immediate future and in view of the proximity of Building 9 to the Clinical Center, the NIH decision to renovate this building is most welcome. Budgetary constraints have forced temporary deferral of plans to move IRP operations to newly constructed facilities on Guam.

Several key personnel and programmatic changes during the past year deserve mention as they reflect shifts in the direction and priorities of IRP activities. Program resources were reorganized to enable the establishment of a new laboratory, the Molecular Genetic Branch, under the leadership of Dr. Robert Lazzarini, which will apply emerging molecular virologic and recombinant DNA techniques to the study of nervous system function and disease. The protracted search for a new Chief of the Laboratory of Neurophysiology culminated successfully in the selection of Dr. Jeffery Barker. Dr. Barker, who served as a medical officer in this laboratory since 1976, will strengthen basic neurobiologic investigations in this group, especially in relation to neurotransmitter physiology. Professor Fritz Buchthal of Copenhagen, has been selected to provide interim direction to the Program's EMG (electromyography) and related neuromuscular disease studies, replacing Dr. King Engel, who has assumed an academic post at the University of Southern California. With the active participation of an outside search committee, efforts to recruit a chief for the newly authorized Communicative Disorders Branch have yielded several promising candidates. Less successful have been our continuing efforts to recruit chiefs for the Positron Emission Tomography (PET) and Epilepsy research programs. Nevertheless, work in both areas has continued to be productive under temporary leadership. The NINCDS purchased ORTEC PET scanner is operational and will soon be augmented by an IRP designed "NEUROPET" scanner which has significantly higher resolution. Studies, now limited by the relatively small quantities of fluorine-18 deoxyglucose available from the Naval Research Laboratory, will be greatly expanded with the arrival in 1983 of the IRP purchased CV 46 cyclotron. Finally, it is noted with great regret the passing of Dr. William Caveness, Chief, Laboratory of Experimental Neurology, whose research at NIH spanned over 15 years and contributed substantially to our understanding of the pathogenesis of epilepsy and to the late sequelae of head injuries.

By all criteria IRP investigator-initiated research has continued to flourish. During the past year 13 projects were initiated, 11 were completed or terminated, and 138 projects remain active. The mix of this broad-based effort remains essentially unchanged. The most extensively supported disciplines in the preclinical neurosciences are (in descending order): virology and immunology, physiology, chemistry, and pharmacology. Support of clinically applied investigations is now focused primarily on demyelinating, metabolic-degenerative, hearing, and neoplastic disorders of the nervous system. There continues to be a roughly equal split between resource allocations for basic and clinically applied studies. Regarding

the latter, 75 clinical protocols are now active, with an average of 25 patients being admitted to each study during the past year. Results of the Program's current research effort are presented in the appended Project Reports and in the Summaries provided by the Chiefs of each of the Program's 16 Laboratories and Branches.

Dissemination of IRP research findings continues to be worldwide. More than 360 scientific articles were published during calendar year 1980. Journals in which these articles appeared in descending order of the number of IRP papers included: Archives of Neurology, Proceedings of the National Academy of Science, Advances in Neurology, Annals of Neurology, Journal of Biological Chemistry, Slow Transmissible Diseases of the Nervous System, Science, and Journal of Neurochemistry. In addition, IRP-authored papers appeared in non-English language journals from a number of foreign countries including France, Sweden, Japan and the USSR. Program scientists also assisted in the rapid promulgation of new research findings by organizing and participating in meetings and workshops on a broad range of topics as well as through informal contacts with scientists from this country and abroad.

Another significant, yet sometimes overlooked, IRP contribution to neurosciences research is the superb opportunity for training in many preclinical and clinical areas. Over the years a high proportion of IRP trainees have gone on to distinguished careers in academic and industrial research both here and abroad. This year some 187 investigators took advantage of training opportunities here as Staff Fellows (47), Medical Staff Fellows (9), Clinical Associates (4), Visiting Fellows (55), Visiting Associates and Scientists (21), Guest Workers (36), and Intergovernmental Personnel Act investigators (15). In addition, a clinical neurology training program for medical students has been initiated.

As a further indication of Program excellence, several IRP scientists received special recognition for their exceptional contributions to research during the past year. Dr. C. Joseph Gibbs, LCNSS, received the American Biology Alumni Award from Catholic University for outstanding professional achievements as well as the Navy Commendation Medal from the Secretary of the Navy for sustained superior contributions to military medicine and distinguished service as a Staff Medical Officer. Dr. Roscoe Brady, DMNB, was selected to receive the 1981 Distinguished Alumni Award from Pennsylvania State University, the highest order of recognition offered by that institution. Drs. Brady, Gibbs and Paul Kornblith, SNB, received the first SSS bonuses under the Civil Service Reform Act of 1978, while Drs. R. Wayne Albers, LNC, and Robert Lazzarini, LMG, received Public Health Service Superior Service Awards. Finally, I would be remiss not to acknowledge the outstanding dedication and assistance provided by Dr. Donald Calne, Clinical Director, and Dr. Richard Irwin, Laboratory Director, in fostering the creative environment so essential to the success of the Intramural Research Program.





## ANNUAL REPORT

October 1, 1980 through September 30, 1981

### Instrumentation and Computer Section, ODIR

National Institute of Neurological and Communicative Disorders and Stroke

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## Annual Report of Instrumentation and Computer Section

National Institute of Neurological and Communicative Disorders and Stroke

October 1, 1980 - September 30, 1981

The Instrumentation and Computer Section provides technical support for investigators by (1) assessing the instrumentation and computer needs of the investigator; (2) designing, developing and constructing special-purpose electronic and mechanical instrumentation and systems not commercially available; (3) designing, specifying and managing laboratory computer systems for data acquisition and processing.

Additional services provided by the Section include consultation on measurement techniques, signal processing, noise and electro-magnetic interference in data measurement systems, and equipment purchases. Several formal and informal courses for investigators are taught by Section personnel; topics include electrical circuit theory, operational amplifier applications, digital logic design, and computer applications.

Due to manpower limitations and economic considerations, the Section is unable to provide the following services: repair of commercial instruments, duplication of off-the-shelf commercially available equipment, and fabrication of non-instrument items (shelves, bookcases, etc.).

When an investigator requires the services of the Section, he first meets with the Section Chief and other personnel as needed to discuss his requirements. On the basis of this meeting, a decision is made as to whether ICS (Instrumentation and Computer Section) will take on the project. If a commercially produced instrument will satisfy the investigator's requirements, he is advised to purchase it. If custom instrumentation is needed, ICS will accept the project unless we lack the appropriate expertise, or our current work backlog is excessive. In these cases the project may be contracted to a private firm, or the investigator may be directed to the Biomedical Engineering and Instrumentation Branch (BEIB).

When the Section Chief or the Assistant to the Chief agree to accept a project, the investigator submits a standard work request form (available from ICS), signed by his Lab Chief. This form will state the nature of the instrument or service requested, and will contain as many details and specifications as the investigator can provide.

The project is then assigned to an engineer, who will confer with the investigator to formulate a set of engineering specifications and a timetable and cost estimate for the project. The ICS does not charge for services, but the investigator will be billed for the cost of the components used. Upon delivery of the completed instrument, a memo is sent to the investigator listing the component costs and asking permission to have the Administrative Officer transfer funds from his CAN to the Section's CAN.



## INSTRUMENTATION

The following are selected instrumentation projects undertaken during the past year. These are chosen from a total of 303 projects, and are representative of the types of electronic instruments and systems developed by the Section:

(1) Patient Activity Monitoring System. The Section has continued to develop the Patient Activity Monitor (PAM) and the support hardware and software which forms the system. The major development this year was the long-awaited availability, just announced, of the high density memories in very small packages which will permit the fabrication of a monitor with 10 days capacity for continuous monitoring at 15 minute intervals. Design of this monitor was completed a year ago, but the memory technology was repeatedly delayed. Development of the new monitor will begin immediately, with production late in 1981.

A major disappointment has been the poor reliability of the hybrid monitors. These devices, built by a small local thickfilm hybrid firm, were constructed using untested chips and poor fabrication techniques. Only 39 of the contracted 50 devices have been delivered so far, and only 20 of these are still working. We have abandoned this technology.

The Section continues to produce the standard 64 hour device for new applications, and to replace outmoded units. More than 100 standard monitors are now in the various laboratories.

(2) Neurological Testing Station. The Section has continued instrumentation development to expand and improve the computer-based neurological testing station designed last year. In addition to improvements to the accelerometers and calibration system, a new touch-pad system was developed for use with some new experiments which require much finer movements than are possible with the existing touch-pads. The new system uses a hand-held, pencil-shaped probe which the subject uses to touch rectangular, conductive targets of varying widths and separations. Target separations as small as 4mm are incorporated in the new system with target widths as small as 1mm. The interface electronics allows the computer to control the experiment and to measure the movement time, reaction time and movement accuracy.

(3) CRT Rotating Display. A rotating display system was developed using two Wavetek waveform generators and a PDP-11/34 computer for use as visual stimuli in experiments on the monkey cortex. The system allows the experimenter to generate grating patterns at variable frequencies and attenuation levels and also to be able to rotate the display through 360° as desired. All parameters of the display can be controlled either manually or by the computer. Extensive protection circuitry was also included to prevent CRT damage in the event of a failure of the waveform generators or the computer.

(4) Exploratory Activity Cage. An activity cage developed last year has proved useful in pharmacological studies of drug effects on the exploratory activity of laboratory mice. The cage employs photocell arrays and a 1-bit microprocessor to monitor transitions from a light area to a dark area within the cage, and to compute total time spent in each area. A larger cage, with improved electronics, is now being tested with laboratory rats. Initial results suggest that rats will be useful in this behavior model if shorter trial times are used to compensate for the reduced exploratory nature of these larger rodents.

(5) Large Formal Display System. A commercial large screen raster-scan CRT display has been interfaced to a PDP LSI-11 microcomputer in order that complex spatial and temporal visual patterns may be presented to monkeys during single unit recording. The interface synchronizes the transfer of commands and data between the computer and the CRT display. This high-speed DMA transfer utilizes the high resolution ( $\sim 1000$  raster lines/frame) of the display, and allows generation of arbitrary spatial frequency patterns, either fixed or drifting, at a flicker-free frame rate of 100 frames/second.

(6) Four-Channel Rat Sleep Scorer. Final development and validation of a single-channel rat sleep scorer were completed last year. The benefits of automated sleep scoring warranted the development of a four-channel instrument. In order to reduce cost, size, and power consumption, the hardware of the single-channel prototype was extensively redesigned. In addition, features were added to improve the man-machine interface so that operator training and operating efficiency would both be improved. The four-channel instrument interfaces directly with a microprocessor-based instrument which monitors, accumulates, formats, and prints the on-line output. On command, the instrument also calculates and prints, for each channel, a statistical summary of twenty study parameters using the data base accumulated during the study.

(7) Hydrogen Electrode Amplifier Array. Local cerebral blood flow in unanesthetized animals may be monitored by the hydrogen ( $H_2$ ) clearance method. An array of implanted platinum electrodes follows the washout of inhaled  $H_2$  gas. The current response of each electrode is converted to a voltage by an amplifier circuit. The electrode polarizing potential is subtracted from this converted voltage and the resulting signal is recorded as the  $H_2$  clearance curve. A six-channel array of amplifier circuits has been developed to perform these two operations. The circuits have high sensitivity, low noise, small size, and are low in cost. The packaging allows expansion to 24 channels for more precise localization of the blood flow measurements.

(8) Rat Rotation Monitor. A rotation monitoring system was developed which counts clockwise and counter-clockwise rotations in rats. The system simultaneously monitors counts from eight rats, and stores the counts for up to 32 intervals which are selectable from 1 to 60 minutes. The device incorporates a novel control concept in which four 1-bit microprocessor control units, using a single program counter and CMOS EPROM, operate in parallel. Parallel processing increases the speed and reduces the memory size. Since memory is the most expensive component in the system, it also reduces the cost.

(9) CSF Valve Control System. A device was developed which controls the sampling rate of experiments which take cerebrospinal fluid from monkeys. The controller uses thumbwheel switches for selecting open and closed times for four channels controlling four independent valves. The device also allows the experimenter to monitor the state of any valve at any moment in time.

(10) Ultrasonic/Infrared Activity Monitor. The development of a system for tracking animals over very large areas was begun. The device will employ a Polaroid ultrasonic ranging system for transducing the animal's distance, mounted on a stepper motor which is processor controlled. Two different system modes are presently being evaluated. In the open loop mode the motor will sweep the allowed range of movement of the animal, noting the animal's location on each sweep. In

the closed loop mode the device will continuously track the animal's movements using either the ultrasonic transducer's output, or the output from an infrared sensitive transducer as the feedback signal. The device can be used to monitor any animal with the restriction that no object can be between the animal and the sensor.

(11) Rat Activity Monitoring System: Solid State Imaging System. A prototype of a system to measure rat activity over a large area was built. The imaging system was an 8x8 array of discrete Darlington phototransistors with their associated bias resistors and amplifiers, mounted on a custom printed circuit board. Tests of the array with a simple double convex lens and a back lighted cage floor, 0.5 meter x 0.5 meter, had very good results. When the cage floor was extended to 1.0 meter x 1.0 meter, it was found that the simple lens introduced too much distortion and that a more complex lens must be used. In addition to the development of this system, the possibility of using a low cost commercial closed circuit video system for large area monitoring is being examined.

(12) Visual Evoked Response Stimulus System. A visual evoked response stimulus system is being developed. It will randomly select one of eighteen 35mm slide images and project it onto a 35cm x 50cm opaque screen with a less than 3 msec rise time. The system will continue to randomly display images with a one second delay between images. The projection system will include a very fast electromechanical shutter (2.3 msec. opening time) and two stepper motor controlled rotating wheels; one will carry 35mm slides and the other will carry neutral density filters.

(13) Multi-Channel Programmable Reinforcement Scheduler. A microprocessor-based instrument was developed which monitors six input channels (levers operated by animals) and provides an output channel of scheduled reinforcement for each input. The scheduled reinforcement is expressed as a percent of time during a given epoch when a potential input will generate reinforcement. Operator programmable parameters are percent reinforcement, reinforcement duration, epoch duration, and epoch count. Inputs/channel/epoch are accumulated and listed on a printer incorporated into the instrument.

(14) Programmable Infusion Pump. A microprocessor-based instrument is being developed which will control a motor driven syringe. Various pumping schedules which have mathematical analogies can be specified by the operator, and cause the pump to run at exponential, ramp, or constant rates. After a schedule is specified by the operator, the instrument prompts the operator for input arguments, calculates certain variables and delivers the pump command.

(15) Animal Activity Monitoring. Two types of detectors are being investigated as passive, non-invasive transducers for measuring animal spatial activity (rearing, gross movement, velocity, etc.) in an enclosed environment. (A) Polyvinylidene fluoride (PVFD) is a synthetic polymeric film that exhibits high levels of pyroelectric activity following a polarization process. This property makes it useful as a thermal detector. Samples of this material have demonstrated reasonable sensitivities and angular resolutions in detecting laboratory animals and humans. (B) Quantum detectors such as mercury cadmium telluride, lead sulfide, and lead selenide are photoconductive materials which exhibit spectral emission sensitivities in the intermediate to far infrared. Radiation from mammals is sufficient in this region to provide detectable signatures for processing. Each of the materials can be configured as a single element, linear array, or quadrant detector and demonstrates a potential for on-line array or image processing of spatial activity.



## COMPUTERS

The Instrumentation and Computer Section (ICS) continues to support the use of the computer as a laboratory instrument. Small computers are used in the individual laboratories for on-line, real-time interaction, process control and data acquisition. ICS maintains support computers in Buildings 10 and 36. These systems provide means for program preparation, bulk storage, printing and plotting, and mathematical and statistical processing. Experimental data may be transmitted from the laboratory computers, via these systems, to the DCRT facilities for further processing. The support computers also serve to develop prototype systems and to test the feasibility of the use of a computer in specific laboratory applications. The latter capability allows an investigator, once he determines that the computer will do the job, to purchase an efficient system at minimal cost. The Section also maintains an image processing system, described below.

The Section provides software support for the individual investigators. A library of procedures has been developed that is tailored to the needs of the Intramural Program. Individual training is available for investigators with no prior experience in using or programming the computer. Computer specialists are available for consultation in all areas of computer use, programming, interfacing, real-time applications, time series analysis, data presentation, systems configuration and computer procurement. Although ICS does not provide an applications programming service, systems have been developed in collaboration with individual laboratories. Examples are included in the list of computer projects.

Program maintenance is an important function of the Section. Programs used in a real-time, interactive laboratory research environment often produce new information which calls for modification of the program before the next experiment. In addition to the software library and research related projects developed by ICS, much work is caused by the turnover of scientific and support personnel. Many systems developed by these persons prove useful to the laboratory. After they leave, maintenance of such systems becomes the responsibility of ICS. Structured programming techniques and standardization on PASCAL have enabled the Section to provide these services without an increase in personnel. There are currently more than 50 minicomputers in the Intramural Program.

The Section also maintains a microprocessor development system for software and hardware development of microprocessor-based instrumentation at both the chip level and the single board computer level. The system currently supports three common microprocessors, one 16-bit processor and two 8-bit processors. Various utility programs and two high level language compilers are available (FORTRAN and PLM) for application programming.

The support computer in Bldg. 36 was upgraded this year, and with the acquisition of two laboratory systems for program development, much of the burden on this facility has been somewhat relieved. However, increasingly sophisticated mathematical algorithms are being developed in the areas of image processing, cell membrane analysis and digital signal processing. These techniques require an increasing amount of processor time, and the existing single user systems are not the most cost effective method of handling these problems.

After a careful system study, a 32-bit computer has been ordered. Space for this facility will be furnished by the Laboratory of Cerebral Metabolism. This computer processes mathematical data more efficiently than any of the existing

16-bit computers and has a time shared, virtual memory operating system. It will have a compatibility mode in which programs written on the existing computers will run with little or no modification. Programs may be written and compiled on this system to be run on the laboratory computers. The two existing image processing systems will be linked directly to this computer, via a high-speed communication linkage. Five computer terminals will be furnished initially. The number of users may be expanded as the need develops. Future plans call for connecting laboratory computers to the facility and develop a true distributed network. This will provide increased capability for the laboratory satellite, at less cost to the user.

One of the major functions of the Section is to provide systems studies for use of the computer as a laboratory instrument. The concept of the mini- and microcomputer as an integral part of laboratory instrumentation for process control, data logging, timing and coordination of instruments has proven to be both cost effective and efficient for laboratory applications. Mini- and microcomputer based systems provide a wide spectrum of tools for research ranging from lower cost data loggers to sophisticated systems which interact with the on-going experiment. These systems have resulted in a further integration of the engineering and computer functions of the Section, and have enabled us to offer a wide range of computer related services. Projects such as the Neurological Testing Station are the result of close coordination between the scientist, the electronic engineers and the computer scientist and illustrates a type of service not feasible a few years ago.

#### IMAGE PROCESSING SYSTEM

The Instrumentation and Computer Section maintains a general purpose image processing system. This system consists of a high-speed rotating drum scanner, an image array processor and display, and a PDP-11/60 computer. The drum scanner can digitize transparencies up to 10x10 inches with spatial resolution of 12.5 microns. The image array processor can simultaneously store, display, and manipulate up to three 512x512 digitized images. Images may be compared, superimposed, translated, zoomed or color coded at video rates. Images to be processed may be obtained by scanning autoradiographs, x-ray film, or photographic negatives, or by using previously digitized images generated by CAT or ECAT scanners. A camera station is being added this year.

An interactive, menu driven, software system was developed to provide an extensive and expandable repertoire of basic image processing and input/output functions. Special purpose functions can be developed to meet specific user requirements. The facility is useful for numerous applications involving evaluation and quantification of biomedical images. Two applications, however, are primary: analysis of two-dimensional electrophoresis gels and analysis of autoradiographs of brain or tissue sections.

The autoradiographs are used for measurements of glucose utilization in brain tissue using the Sokoloff deoxyglucose method of glucose substitution. Analysis of the autoradiographs involves displaying the digitized image in a TV monitor and outlining areas of interest. The average optical density is then computed and automatically converted to glucose utilization. Glucose utilization of brain regions as small as 100 microns in diameter can be computed. A color coded glucose utilization map may also be produced.

Measurement of amino acid concentrations can be made using two-dimensional electrophoreses gels. The gels, which have been prepared by the appropriate

stain and fixer, are photographed; or if radioisotopes are used, an autoradiograph is obtained. The film is scanned and digitized into an array of optical density within a defined boundary. A test gel may be compared with a standard gel using the image array processor to determine the presence or absence of a particular substance.

Additional examples of computer projects include:

(1) Membrane Activity of Neurosecretory Cells. This system was developed in collaboration with the Laboratory of Neurophysiology (NINCDS) to study the conductance of cell membranes that often exhibit bursting activity during clamping. The system is being upgraded to record and monitor the tissue culture neurons on-line and to allow the iontophoretic or pressure injection of neuroactive substances onto the external surfaces of the cells. The purpose is to discover the nature of the action of such substances on the properties of the cell membrane.

(2) Cell Culture Analysis. This system is designed to provide an on-line analysis of tissue culture neurons. The first phase, to study the excitatory or inhibitory post-synaptic potentials of these cells, has been completed. A unique feature of this system is the on-line control of artifacts introduced by the measurement system and the properties of tissues in culture and to control the threshold levels and amplification level as the experiment is in progress. Visual displays of amplitude, integral and latency are available, as well as averaged evoked response. In addition, on-line monitoring of post-synaptic potentials elicited by stimuli presented in pairs or in trains of pulses are available. The system also studies spontaneously occurring miniature potentials. This system will be extended to allow analysis of the cells by other techniques such as voltage clamping and the iontophoretic injection of neuroactive substances on the surface of the cell.

(3) Membrane Channel Analysis. Acetylcholine molecules outside neural membranes open and close channels allowing the passage of  $\text{Na}^+$  and  $\text{K}^+$  ions. Current fluctuations resulting from single channels opening and closing have a high signal-to-noise ratio. This program catalogues and identifies the duration and amplitude of channel activity, and provides a spectral distribution of the electrical activity of the membrane.

(4) Cytofluorographic Analysis of Single Cells. The system designed for cytofluorographic cell sorting by the Computer Systems Laboratory, DCRT, was modified for the cytofluorographic analysis of cells in immunological studies.

(5) Analysis of Data From the HP8450 Spectrophotometer. This highly versatile spectrophotometer can communicate with a computer. It may run under its own program control or under the control of the computer, but its data processing and storage capabilities are limited. A series of routines to control the HP, process and store the data in a form suitable for future analysis or act as a simple data logger, has been developed.

(6) Activity Analysis Software System. A software system was developed which supports the analysis and management of activity data, both human and animal. The system allows one to produce raster plots of activity as well as sophisticated statistical analysis of activity data accumulated over long time periods. Data rates from 1 to 20 samples per hour can be accommodated. The software interfaces presently with two kinds of data acquisition systems. The programs are implemented using the PASCAL language.

# ENGINEERING, COMPUTER AND FABRICATION SERVICES

This table shows the distribution of the Section's workload among the various laboratories and branches.

<u>LABORATORY OR BRANCH</u>	<u>HOURS</u>	<u>PERCENT</u>
Adult Psychiatry, NIMH - - - - -	3481	11.21
Neurophysiology, NIMH - - - - -	2864	9.22
Biological Psychiatry, NIMH - - - - -	2649	8.52
Neuropsychology, NIMH - - - - -	2247	7.22
Clinical Science, NIMH - - - - -	2067	6.65
Neurochemistry, NINCDS - - - - -	1797	5.78
General and Comparative Biochemistry, NIMH - - - - -	1770	5.69
Molecular Biology, NINCDS - - - - -	1509	4.86
Neuropathology and Neuroanatomical Sciences, NINCDS - - -	1440	4.63
Cerebral Metabolism, NIMH - - - - -	1407	4.52
Neurophysiology, NINCDS - - - - -	1256	4.04
Biophysics, NINCDS - - - - -	1064	3.42
Office of the Director, IRP, NINCDS - - - - -	840	2.71
Psychobiology, NIMH - - - - -	731	2.35
Brain Evolution and Behavior, NIMH - - - - -	688	2.21
Infectious Diseases, NINCDS - - - - -	606	1.95
Central Nervous System Studies, NINCDS - - - - -	337	1.08
Surgical Neurology, NINCDS - - - - -	334	1.07
Neuro-Otolaryngology, NINCDS - - - - -	308	.99
Neurochemistry, NIMH - - - - -	298	.96
Vision Research, NIMH - - - - -	288	.93
Experimental Therapeutics, NINCDS - - - - -	206	.66
Clinical Neurosciences, NINCDS - - - - -	199	.64
Psychology and Psychopathology, NIMH - - - - -	185	.60
Neural Control, NINCDS - - - - -	98	.32
Neuroimmunology, NINCDS - - - - -	96	.31
 NIMH (Total)	 18,676	 60.09
 NINCDS (Total)	 10,090	 32.47
 NICHD (Total)*	 2,313	 7.44
<hr/>		
	31,079	100.00

\*NICHD loans the Section one position, and is thus entitled to 1700 hours of service.









# ANNUAL REPORT

October 1, 1980 through September 30, 1981

Neuroepidemiology Section, ODIR  
National Institute of Neurological and Communicative Disorders and Stroke

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Annual Report  
for Period October 1, 1980 through September 30, 1981  
Neuroepidemiology Section  
Office of the Director  
Intramural Research Program  
National Institute of Neurological and Communicative  
Disorders and Stroke

Bruce S. Schoenberg, M.D., Dr.P.H., Chief

The Neuroepidemiology Section is responsible for the development and implementation of epidemiologic and genetic programs to investigate the cause, prevention, and treatment of neurologic disorders in human populations. Emphasis has been placed on major neurologic diseases in which the diagnoses can be clinically verified to the satisfaction of skilled neurologists.

The Section is unique in being the only unit devoted exclusively to research in the epidemiology of diseases of the nervous system. These research studies require collaboration of many individuals. However, since there is a severe shortage of available manpower in neuroepidemiology, the Section developed an active teaching program for current and future collaborative investigators. A series of four videotapes produced by the Section are distributed on a loan basis without charge. A textbook, entitled NEUROLOGICAL EPIDEMIOLOGY: PRINCIPLES AND CLINICAL APPLICATIONS, was published, and a scientific quarterly journal entitled NEUROEPIDEMIOLOGY had been initiated. A symposium on the solutions to methodologic problems in neuroepidemiology was held in conjunction with the Society for Epidemiologic Research and the World Federation of Neurology. In cooperation with the World Health Organization and the World Federation of Neurology Research Committee on Neuroepidemiology, formal courses were conducted in Beijing, China; Madrid, Spain; and Florence, Italy. Future workshops and symposia are planned in collaboration with the American Academy of Neurology, the World Health Organization, the World Federation of Neurology, and the International Epidemiological Association. These sessions serve as a stimulus for neuroepidemiologic research on a worldwide basis. We are also providing opportunities for fellows to spend from six months to one year working with members of the Section in order to learn the techniques of neuroepidemiology. During the past year we have had physicians from Great Britain, Nigeria, Mexico, and Turkey, and have received inquiries from China, Spain, and Israel for future opportunities. There is considerable neuroepidemiologic interest among senior neurologists (two of the physicians working in the Section are professors and chairmen of their own units abroad). Finally, current individual and institutional research training grant programs have recently been

expanded to include neuroepidemiology. With the initiation of an educational program, the Section has focused on research investigations.

Epidemiologic studies have two basic requirements: uniformity and accuracy of data collection. This necessitates the use of a standardized, internationally accepted classification and coding system. The most recent scheme generated by the World Health Organization is seriously deficient with regard to neurologic disorders. The Section is therefore collaborating with the World Health Organization Neurosciences Program, the World Federation of Neurology, and the American Academy of Neurology to revise this system of classification and improve its usefulness for neuroepidemiologic research.

Another important problem for the neuroepidemiologist is the enormous cost of maintaining neurologic surveillance on a large number of patients. Therefore, we have attempted to utilize existing registries of neurologic disease, such as in a study of presenile dementia based on the Israeli National Neurologic Disease Registry. In addition, we are in the process of organizing information routinely collected through the British National Health Service on all neurologic inpatients in a section of London with a population of 3-1/2 million inhabitants. The utility and accuracy of these data have been demonstrated in ongoing studies of the Guillain-Barré syndrome. A similar registry has been organized for the population of northeastern Italy.

There have been a number of neuroepidemiologic case-control studies which have suggested associations between a given factor and a particular disease, but the number of patients has been inadequate for meaningful conclusions. We are working in collaboration with a number of multiple sclerosis clinics to establish a uniform protocol and data base to enable us to explore several hypotheses of interest which require a large number of cases. Similar arrangements are being made to initiate analytic epidemiologic studies of Alzheimer's disease. These several projects in support of research activities, have been initiated in conjunction with a very active research program.

With regard to neurologic problems in children, the Section documented the frequency of primary intracranial neoplasms in the pediatric population of Rochester, Minnesota, and the State of Connecticut. In addition, we investigated cerebrovascular disease in infants and children. The magnitude of this problem was documented for the first time. The study demonstrated that neonatal intracranial hemorrhage is relatively common (1.1 cases/1,000 live births), that it is strongly associated with prematurity and hyaline membrane disease, and that it is difficult to recognize clinically. For pediatric cerebrovascular disease unassociated with birth, trauma, or infection, the incidence rate was 2.5/100,000/year.

These cases were further characterized by survival, residual disability, and cause (whenever possible). The clinical and angiographic features of children with moyamoya disease were examined in detail. This condition appears to be more common than suggested by early case reports. The Section is also studying cerebral palsy in a defined population, to determine if new developments in perinatal care have resulted in changes in the incidence or clinical pattern of these disorders. Our study demonstrated that in recent years, the overall incidence of cerebral palsy has declined. This trend has been especially marked for the spastic syndromes, with the exception of spastic diplegia which has remained relatively constant. Analytic studies are planned to determine risk factors for these conditions.

The Section has conducted extensive investigations of primary intracranial neoplasms. First, problems with nomenclature and disease definition were resolved. After this, two patterns of age-specific incidence emerged. Analyses of most population-based data worldwide revealed a small childhood peak, followed by a later peak between ages 50 and 80. Data for Rochester, Minnesota, however, showed the childhood peak, followed by an increasing incidence rate with increasing age. Careful study of this discrepancy showed 1) that the greater percentage of cases first diagnosed at autopsy in Rochester accounted in large part for this difference, and 2) that a substantial number of brain tumors remain undiagnosed in the elderly during life. Studies have just been completed to evaluate the role of computerized tomography in the diagnosis of brain tumors and to explain the recent increase in the incidence of pituitary tumors among women of child-bearing age. The introduction of computerized tomography has not resulted in any increase in the reported frequency of these tumors in the Rochester, Minnesota population, while the apparent rise in the incidence of pituitary tumors seems to be the result of more sophisticated neuroendocrine diagnostic procedures. An exhaustive, critical review of a survey strategy to measure the national incidence and prevalence of intracranial neoplasms has been completed. In addition, racial differentials in the frequency of certain intracranial tumors (meningiomas and pituitary adenomas) are being examined. Investigations of the relationship between intracranial neoplasms and extracranial tumors have been especially rewarding. An association was found between the occurrence of breast cancer and meningioma in women. This result raises interesting etiologic possibilities when considered with other evidence: 1) meningioma is the only common intracranial neoplasm with a higher incidence in females; 2) the abrupt clinical appearance or enlargement of this tumor during pregnancy has been described; and 3) the finding of estrogen receptor protein in meningioma has been reported.

At the present time, there is little to suggest that improved medical management of the completed stroke will



substantially affect the cerebrovascular disease problem. It would appear that greater benefit could be achieved by dealing with the precursors of stroke rather than delaying treatment until after the event has occurred. Therefore, a non-concurrent, prospective study of a cohort of 2,000 elderly individuals was undertaken to determine the role of heart disease and hypertension as risk factors for both transient ischemic attacks and completed stroke. When the case-control approach was applied to these data, different patterns of risk factors were demonstrated for transient ischemic attacks and completed ischemic stroke. While hypertension, diabetes mellitus, definite hypertensive heart disease, and valvular heart disease are important risk factors for completed ischemic stroke, these disorders have a substantial effect on the subsequent risk of TIA. When these data were analyzed in the format of a prospective study, it was possible to calculate the absolute risk of stroke as a function of the presence or absence of specific forms of cardiovascular disease. The following types of cardiovascular disease yielded the highest ischemic stroke incidence rates (given in cases/1,000/year): myocardial infarction (15.5); congestive heart failure (20.5); and TIA (42.0). In considering risk factors for TIA, both angina/coronary insufficiency and congestive heart failure yielded the highest rates (10.4 and 10.9, respectively). Once etiologic precursors of stroke have been identified, medical intervention before the occurrence of long-lasting disability requires that there be an interval of time between the onset of the risk factor and the development of completed stroke. Analysis of data from this non-concurrent prospective study revealed that those developing borderline hypertension, valvular heart disease, or ischemic heart disease remained stroke-free for the initial one and one-half years after the first occurrence of each specific form of cardiovascular disease. This finding implies that there is an interval of time following the onset of these conditions when it may be possible to intervene medically to reduce the risk of stroke.

Other investigations in the area of stroke involve a careful analysis of unusual patterns of cerebrovascular disease (e.g., more than 20 TIA's/day).

Alzheimer's disease/senile dementia, despite its high apparent clinical frequency among the elderly, has not been well studied in a U.S. population. Because of this, we have launched three investigations. One is derived from a review of detailed clinical records utilizing a population-based, records-linkage system; the second utilizes a two-stage survey consisting of a questionnaire and clinical examination; and the third (in collaboration with the National Institute on Aging) is based on a questionnaire survey. In the records-linkage study, a neurologist using fixed diagnostic criteria reviewed records from all medical facilities serving the residents of Rochester, Minnesota. This made it possible for the first time to determine the incidence of dementia

coming to medical attention in a well-defined U.S. population. For those age 30 plus, the incidence rate was 110 new cases/100,000 population/year. The rates increase with age, and the age-specific rates were higher in women. To confirm the reduced survival of demented patients reported on the basis of individuals hospitalized at specific medical centers, we examined the survival of all demented individuals identified through our records-linkage study. Dealing with an entire population minimizes any possible selection bias that may be present for a series of patients seen at a particular medical institution. The survival rates generated for all demented patients in the defined population were significantly reduced compared to age- and sex- matched survival statistics derived from life-tables for residents of the northwest central region of the U.S., thereby documenting in a community study previous observations based on hospitalized patients.

The two-stage survey permitted us to estimate the prevalence of dementia in a biracial community. For each race, prevalence ratios were higher for females. For each race and sex, the prevalence figures rise dramatically with age. This morbidity study indicates that dementia represents a major health problem for both racial groups.

There has been some debate as to whether Alzheimer's disease is a single disease entity regardless of its age at presentation. Since the frequency of Alzheimer's disease is relatively low before age 60, an enormous population is required for surveillance in order to obtain an adequate number of patients for study. We are therefore utilizing the resources available through the Israeli National Neurologic Disease Registry to identify all potential cases among the population of Israel. These cases will be intensively reviewed to determine the accuracy of diagnosis and to explore a number of epidemiologic studies of the distribution and risk factors for this disease. A similar sex-ratio for patients with onset before and after age 60, and a steadily increasing age-specific incidence in the elderly would argue in favor of a single disease entity.

The Section is also interested in accurately documenting possible racial differentials in the prevalence of major neurologic disorders. A number of early investigations suggested possible differences by race, but were based on hospital or clinic experience and could not identify a well-defined population from which cases were derived. Population-based studies followed, but questions concerning the results centered on possible racial differentials in access to expertise in neurologic diagnosis and treatment. We reinvestigated (in conjunction with the Surveys and Demographic Studies Section) this problem of possible racial differentials in the prevalence of major neurologic disorders by surveying a well-defined population (approximately 25,000, almost equally divided between blacks and whites). We



developed a strategy which eliminated the requirement that persons must have entered the health-care system for detection of disease. The disorders investigated included cerebral palsy, dementia, psychomotor delay, epilepsy, Parkinson's disease, essential tremor, and cerebrovascular disease (both transient ischemic attacks and completed stroke). The basis of the investigation was a door-to-door survey which utilized a detailed questionnaire inquiring not only about diagnoses, but also about signs and symptoms suggestive of neurologic dysfunction. Over 99% of the households agreed to the interview. Those household members suspected of having one of the disorders of interest were then asked to have a neurologic examination conducted by a senior, board-certified neurologist. The interviews and examinations have been completed, and the data are being edited and analyzed. Data currently available for Parkinson's disease indicate that in the population studied, parkinsonism is more common in whites but the difference between races is not as great as suggested by earlier studies. The same survey yielded information on essential tremor, thereby providing the first data on the prevalence of this condition in a defined U.S. population. For either race, the prevalence ratios was slightly greater in women, and for either sex, the figures were slightly higher for whites.

Similar strategies are being developed for application in developing countries (e.g., Nigeria, Mexico, Turkey, and the People's Republic of China), in collaboration with the World Health Organization.

We currently have very little information on the patterns of medical care received by all individuals with neurologic disease in a given community. The Section is, therefore, studying this problem in Rochester, Minnesota. Although the findings of this investigation will not necessarily be applicable to other regions of the U.S., the City of Rochester does offer particular advantages. Cases of neurologic disease among residents have already been identified through previous studies. Medical encounters are easily documented through a records-linkage resource. In addition, Rochester residents have access to high-quality medical care, and physicians with neurologic expertise are available within the community. Thus, the Rochester experience may provide some estimate of the pattern of medical care in the ideal situation in which the population has ready access to neurologic expertise, and in which there is little financial restraint to such care. The study for patients with brain tumor is being prepared for publication, and similar data are being analyzed for completed stroke.

Although death certificate data are limited by possible misdiagnosis, incomplete case ascertainment, errors in coding, etc., detailed morbidity information on neurologic diseases for the entire U.S. and for other countries is not available. The Section has analyzed mortality data for

selected neurologic disorders by country and by county in the U.S. The overall patterns which emerge may be useful in evaluating trends over time and in formulating etiologic hypotheses. Among the most interesting findings is that the mortality from cerebrovascular disease has decreased in most developed countries over a 20-year period. This trend is not universal, however. For multiple sclerosis, countries initially reporting high mortality rates have generally reported declines while those with low rates earlier are reporting increases, so that more recent mortality data for multiple sclerosis by country show less of a differential than previously reported.

A number of other collaborative projects include the investigation of space/time clusters of neurologic disease (with the Center for Disease Control), the development of survey strategies (with the World Health Organization and the Section on Disease Statistics Surveys), a study of myasthenia gravis and multiple sclerosis in the same patient (with the Mayo Clinic), an investigation of neurologic disorders during pregnancy and the postpartum period (with the Mayo Clinic), a study of the epidemiology of eye tumors (with the Connecticut State Department of Health), the effect of weather on the incidence of stroke (with the Mayo Clinic), and international comparisons in the incidence of brain tumors. Finally, extensive reviews have been prepared on the epidemiologic aspects of Huntington's disease, otitis media, Alzheimer's disease, cerebrovascular disease, primary intracranial tumors, Tourette's syndrome, peripheral neuropathy, neurologic diseases in the elderly, and controlled therapeutic trials of motor neuron disease.

The clinical neurogenetics component of the program involves three areas: 1) genetic-epidemiologic studies of movement disorders (e.g., the dystonias); 2) genetic-epidemiologic studies of multifactorial neurologic disorders (e.g., Parkinson's disease and multiple sclerosis); and 3) genetic and biochemical studies of hereditary nervous system tumors.

Our contribution of most immediate clinical application involves documentation in the United States of a distinct, treatable form of myoclonus epilepsy. Based on a study of 27 patients in 15 families, we describe a form clearly distinct from Lafora body disease. Because this condition appears to be the same as that originally described in Estonian families by Unverricht and Swedish families by Lundborg, and more recently by several groups of investigators in over 80 families in Finland, we term the disorder "Baltic" myoclonus epilepsy. Recognition of this entity is crucial since use of phenytoin, which has been routine, was associated with death in 9 of our cases. In contrast, termination of phenytoin and use of valproate sodium was associated with marked improvement in the 7 cases employing this approach. The harmful effect of phenytoin remains unproven, but the longer, more moderate

course in those born prior to use of phenytoin and similar results to ours in over 20 cases in Finland support this critical notion.

In the area of multifactorial disease, we have now ascertained over 165 twin pairs and one set of quadruplets with parkinsonism. Clinical and genetic study of 36 monozygotic twin pairs and 17 dizygotic twin pairs, selected on the basis of at least one member being diagnosed as having Parkinson's disease revealed only one monozygotic pair concordant for the disease and none of the DZ group. Although the unaffected co-twin in each case remains at risk, this very low concordance suggests that neither typical environmental nor genetic factors are critical determinants. Data on smoking support an earlier impression that there is a decreased risk for Parkinson's disease in smokers. Analysis of clinical and psychological observation and interview data on 25 MZ twin pairs discordant for Parkinson's is underway. If life-long differences in personality are present in affected versus unaffected twins, as our preliminary study suggested, a very early determinant for Parkinson's disease operative even in the prenatal period, is possible.

Three surviving quadruplets, one of whom has Parkinson's disease, have been extensively evaluated neurologically and psychologically. They, too, appear to show the same life-long differences in personality as do the discordant monozygotic twins.

Over 140 MS twin pairs have been ascertained. Genetic analysis of 51 pairs personally examined indicates high concordance in MZ twins with 8 of 22 both affected compared to 3 of 29 dizygotic twin pairs. This suggests a significant genetic contribution as well as an environmental component. The fact that all 11 of the concordant twin pairs for MS are female compared to a female/male ratio of MS of 6:4 in the general population suggests that genetic factors may be sex-influenced. For this reason, when evaluating data on multiple sclerosis, male cases and female cases should be considered separately.

Studies in the area of hereditary tumors of the nervous system have involved central neurofibromatosis with bilateral acoustic neuroma, primarily. Efforts have been directed at improving diagnosis, particularly in the early stages in this disorder of relatively late onset, either through clinical means or by linkage analysis. Clinical studies include evaluation of auditory-evoked response and CAT scan with and without gas insufflation as a means of early documentation of acoustic neuroma and following change in tumor size with and without intervention.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 01924-11 ODIR																																						
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SECTION Clinical Neurogenetics Studies, Neuroepidemiology Section																																								
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																																								
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SUMMARY OF WORK (200 words or less - underline keywords)  <p>In this project, we seek to 1) clarify and expand the nosology of the <u>hereditary movement disorders</u>; 2) contribute to the understanding of their <u>underlying biochemical basis</u>; 3) determine the most effective treatment for each disorder; and 4) suggest guidelines for <u>counseling individuals at risk</u>. General syndromes under study include the <u>dystonias, tic disorders, Huntington's chorea, and myoclonus</u>. Approaches include standard epidemiologic and clinical genetic studies together with collaborative efforts in evaluating the role of neurotransmitters such as dopamine, their precursors, and metabolites, and their necessary cofactors.</p> <p>Former title: (Genetic Studies of the Torsion Dystonias and Other Disorders of Movement.)</p>																																								



## Project Description:

Objectives: Included among the disorders of movement such as the choreas, the dystonias, and tic syndromes are a number of discrete diseases which are due to a single gene mutation. Examples of mutations producing autosomal dominant traits are Huntington's chorea and one form of torsion dystonia. Examples of mutations leading to autosomal recessive traits are Lafora type myoclonic epilepsy, the newly described Baltic type myoclonus epilepsy, and the type of torsion dystonia responsible for most cases of dystonia in the Jewish population.

In this project we seek to 1) uncover additional specific diseases within general movement disorder syndromes; 2) contribute to the understanding of their underlying biochemical basis; 3) determine the most effective treatment for each disorder; and 4) suggest guidelines for counseling individual family members.

Methods Employed: Initially, families with members exhibiting a particular syndrome undergo detailed clinical evaluation. Extensive genealogic data are then analyzed in conjunction with clinical observations and relevant laboratory studies. A nosologic classification is prepared. Promising biochemical leads are explored in collaboration with established investigators. Simultaneously, existing treatment programs are evaluated, and where indicated, there are therapeutic trials of new agents.

Major Findings: During the past year, a comprehensive review has been prepared concerning over 40 hereditary movement disorder syndromes. The review was based on a personal study of over 500 selected families evaluated by us. The review includes description of two diseases delineated by us: 1) the recessive form of torsion dystonia, and 2) the Baltic form of myoclonic epilepsy. In addition, we have reported finding low hydroxylase cofactor in spinal fluid of six relatives with familial dystonia.

Significance to Biochemical Research and the Program of the Institute: Individually these disorders are not common but collectively the hereditary disorders of movement represent one of the major public health problems. In addition, information gained from analysis of these discrete genetic traits may contribute understanding to the cause and treatment of more common problems, such as parkinsonism, in which the genetic constitution may be only one of several contributing factors.

Proposed Course: Continue search for distinct entities within movement disorders syndromes seeking their biochemical basis, specific therapy and prevention. Investigations of a screening test for cofactor alteration in urine in various forms of dystonia and an evaluation of specific therapy in Baltic myoclonus epilepsy are planned.

Honors and Awards

Lecturer, Division of Medical Genetics, Columbia Medical Center, New York, N.Y.

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COOPERATING UNITS (if any) SN, IRP, NINCDS; OP, DR, CC; LVC, NCI Department of Medicine, Johns Hopkins Hospital																																						
LAB/BRANCH Office of the Director, Intramural Research Program																																						
SECTION Clinical Neurogenetics Studies, Neuroepidemiology Section																																						
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																																						
TOTAL MANYEARS: <div style="text-align: center;">0.75</div>	PROFESSIONAL: <div style="text-align: center;">0.25</div>	OTHER: <div style="text-align: center;">0.5</div>																																				
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <span><input checked="" type="checkbox"/> (a) HUMAN SUBJECTS</span> <span><input checked="" type="checkbox"/> (b) HUMAN TISSUES</span> <span><input type="checkbox"/> (c) NEITHER</span> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <span><input type="checkbox"/> (a1) MINORS</span> <span><input type="checkbox"/> (a2) INTERVIEWS</span> </div>																																						
SUMMARY OF WORK (200 words or less - underline keywords)  <p>In this project we seek: to define and classify <u>hereditary tumors of the nervous system</u>, in addition to the nine such diseases already recognized; to add to the <u>clinical description</u> and <u>natural history</u> of these diseases; to suggest methods for <u>early diagnosis</u>; <u>evaluate present modes of treatment</u>; and develop methods for <u>preclinical detection</u> and screening.</p>																																						

## Project Description:

Objectives: There are at least nine genetically determined syndromes which include as one of their chief manifestations tumors of the nervous system. Peripheral neurofibromatosis and tuberous sclerosis are among the more common examples. It is the objective of this project to document additional hereditary traits which can cause such neoplasms; add information to the clinical description and natural history of such traits; suggest effective means of early diagnosis; evaluate various modes of treatment and develop methods of preclinical detection and screening.

Methods Employed: In families with two or more individuals affected with the same rare tumor of the nervous system, members undergo clinical, genealogic and radiologic evaluation. Appropriate physiologic and biochemical studies are carried out in collaboration with laboratory investigators.

Major Findings: We have documented the existence of central neurofibromatosis with bilateral acoustic neuroma. Recently, we have reported on clinical and genetic findings in over 130 individuals with this trait.

In collaboration with the Department of Medicine, Johns Hopkins Hospital, and the Laboratory of Viral Carcinogenesis, NCI, we have evaluated the usefulness of nerve growth factor in serum as a means of preclinical detection. To date, nerve growth factor has been evaluated in 30 affected individuals and their relatives from three kindreds previously studied by us. A major need of the nerve growth factor assay is to improve its reproducibility, particularly at low concentrations.

Recently, we have embarked on a study to determine the usefulness of CAT scan with CO<sub>2</sub> in diagnosis and management of bilateral acoustic neuromas. Neurosurgical and neuroradiologic colleagues are participating.

Significance to Biochemical Research and the Program of the Institute: Hereditary tumors of the central nervous system are generally treatable if diagnosed early. Radiologic and physiologic techniques permitting early diagnosis would be of great use. Since many of these hereditary tumors are autosomal dominant in their inheritance pattern with onset during or after the childbearing years, there are individuals in such kindreds who carry a 50 percent risk of developing the trait who are faced with the question of family planning.



Such individuals would gain immediate benefit if reliable, noninvasive, predictive tests were developed. Also, knowledge gained in the course of this practical application should contribute to understanding the mechanisms of tumor development.

Proposed Course: We have reported our clinical and genetic findings in 130 affected individuals with central neurofibromatosis with bilateral acoustic neuroma. The utility of nerve growth factor and other growth factors as preclinical detectors in the individuals at risk requires clarification.

Comprehensive screening programs as well as genetic linkage analysis for neurofibromatosis, von Hippel-Lindau syndrome and other hereditary nervous system tumors will be undertaken when resources permit.

#### Publications:

Eldridge, R.: Central neurofibromatosis with bilateral acoustic neuroma. In Riccardi, V.M., and Mulvihill, J.J. (Eds.): Advances in Neurology. New York, Raven Press, 1981, pp. 57-65.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02167-07 ODIR																																										
PERIOD COVERED <b>October 1, 1980 through September 30, 1981</b>																																												
TITLE OF PROJECT (80 characters or less) <b>Genetic Epidemiology Studies in MS and Other Multifactorial Neurologic Disorders</b>																																												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																																												
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	Donald Calne	Chief		ET IRP	NINCDS																																							
	James Dambrosia	Mathematical Statistician	OBFS	OD	NINCDS																																							
COOPERATING UNITS (if any) Department of Neurology, University of Oregon Department of Neurology, Rutgers University, Piscataway, N.J. ET, NI, IRP and OBFS, OD, NINCDS																																												
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SUMMARY OF WORK (200 words or less - underline keywords)																																												
<p>In this project we are coupling genetic study of <u>selected families</u> and twin pairs with epidemiologic, immunologic, serologic and neurochemical studies of disorders due to <u>multiple factors</u> such as <u>multiple sclerosis</u> and <u>parkinsonism</u>. This approach should <u>clarify the etiology</u> of these diseases, indicate individuals or populations at <u>high risk</u> and suggest a mechanism for prevention and treatment.</p> <p>To date, over 20 presumptive "<u>Multiple Sclerosis</u>" families and over 140 twin pairs with this condition have been ascertained. Over 165 twin pairs with <u>parkinsonism</u> have been ascertained.</p>																																												

## Project Description:

Objectives: As the genetic control of immune response becomes clarified, new avenues of exploring diseases such as multiple sclerosis (MS) are suggested. Improved understanding of the chemical and cellular changes underlying parkinsonism also permits new approaches to its study. Our objective is to couple this new understanding with genetic and epidemiologic techniques in order to clarify disease mechanism, indicate high-risk individuals and populations, and suggest possible means for prevention and treatment.

Methods Employed: Formal techniques of clinical genetics, neurochemistry, serology, and immunology will be merged. Selected populations including families with multiple members or twin pairs affected with the disease will be studied in depth. Unaffected family members, unaffected twins, and spouses will serve as controls. Specific investigations may include: detailed history and neurologic examination, computerized axial tomography; dermatoglyphic analysis; genotyping of blood for red cell antigens, serum proteins and the A, B, and D loci of the major histocompatibility complex; serum studies of viral antibody, immunoglobulin levels and complement levels; spinal fluid examination for routine elements plus determination of immunoglobulin content, oligoclonal banding, presence of myelin basic protein; and cellular study of migration inhibition and mixed lymphocyte culture response and genotyping of "B" lymphocyte, and psychological study including cognitive testing, and objective personality tests.

Major Findings: Most impressive has been the difficulty in ascertaining bonafide MS families, and twins with either MS or parkinsonism. Given the frequency of twinning and the frequencies of MS and parkinsonism, over 1,000 twin pairs with each disorder would be predicted in the United States. Utilizing a variety of ascertainment techniques including patient and physician contact, notices in medical and lay publications, and base-twin registries, less than one-fifth of the predicted number of twin pairs have been found for each condition.

Our detailed study of 14 MS families and 30 MS twin pairs has resulted in the following preliminary conclusions. In half of the families and several of the twins, diagnosis of MS could not be confirmed clinically. Thus, careful clinical documentation is an essential prerequisite in any patient-based study of this disorder. No consistent segregation of HLA type was noted between affected and unaffected family members. Thus, there is not a single, major gene with the HLA complex whose presence is sufficient and necessary for the development of MS.

Of 24 MS twin pairs, 6 were MZ concordant, 6 were MZ discordant, 2 were DZ concordant, and 10 were DZ discordant. All concordant pairs are female. This increased concordance rate in female MZ twins suggests genetic factors are important - but in association with certain sex-influenced environmental events.

A preliminary report based on 12 monozygotic twin pairs discordant for parkinsonism has been presented. Affected twins, in general, smoked less and had more introverted personalities than their unaffected cotwins. A significant genetic contribution has not been supported by evaluation of a further 40 cases.

Significance to Biomedical Research and the Program of the Institute: Disorders in which both genetic and environmental factors presumably contribute, such as MS, comprise a major neurologic public health problem. Ample evidence from data based on populations already indicates genetic factors have a role in causation of MS. By coupling existing knowledge of genetics, the immune response, and neurochemistry, understanding of this group of disorders should be advanced, methods for prevention and treatment suggested and the risk for these diseases in close relatives assigned more accurately.

Proposed Course: Ascertainment of MS families, MS twin pairs, and Parkinson twin pairs continues. The first phase of the MS family and twin studies is nearing completion. A presentation of the clinical and laboratory observations based on 30 MS twin pairs is in preparation. Genetic and epidemiologic reports based on 56 twin pairs will follow.

The second phase of these studies will focus on appropriate epidemiology and laboratory studies in selected genetic groups in which the MS or Parkinson phenotype can be assigned definitely. Presentation of the clinical laboratory observations based on 53 Parkinson twin pairs are in preparation. Preparation of clinical and psychological observation and review data on 25 MZ twin pairs definitely discordant for parkinsonism is in preparation.

Ascertainment of twins with Alzheimer's disease is underway.

#### Publications:

Duvoisin, R.C., Eldridge, R., Williams, A., Nutt, J.D., and Calne, D.B.: Twin study of Parkinson disease. Neurology 31: 77-80, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02240-05 ODIR
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Epidemiology of Dementia		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: Bruce S. Schoenberg      Chief      NES      ODIR      NINCDS		
COOPERATING UNITS (if any)      Epidemiology, Demography, and Biometry, NIA; W. Massey, M.D., Duke University; E. Kokman, M.D. and J.P. Whisnant, M.D., Mayo Clinic; B. Jordan, Harvard Medical School; M. Alter, Temple Univ.; E. Kahana, Hadassah Hospital, Jerusalem, Israel		
LAB/BRANCH Office of the Director, Intramural Research Program		
SECTION Neuroepidemiology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2.5	PROFESSIONAL: 2.5	OTHER:
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  A number of different approaches are being utilized to estimate the <u>mortality</u> and <u>morbidity</u> of <u>Alzheimer's disease/senile dementia</u> in several population groups in the U.S. and to measure the <u>distribution</u> of this disease in segments of the population.		



## Project Description:

Objectives: To obtain estimates of the magnitude and distribution of Alzheimer's disease/senile dementia in segments of the U.S. population.

Methods Employed: Mortality information is obtained from death certificate data for the U.S. Four morbidity studies are also currently underway. One is derived from a review of detailed clinical records utilizing a population-based, records-linkage system; the second utilizes a two-stage survey consisting of a questionnaire and clinical examination; the third (in collaboration with the National Institute on Aging) is based on a questionnaire survey; and the fourth uses records available from a population-based registry serving an entire county.

Major Findings: The mortality data reveal an increasing death rate with increasing age, but probably represent underascertainment to a great extent. In the records-linkage study, a neurologist using fixed diagnostic criteria, reviewed records from all medical facilities serving the residents of Rochester, Minnesota. This made it possible for the first time to determine the incidence of dementia coming to medical attention in a well-defined U.S. population. For those age 30+, the incidence rate was 110 new cases/100,000 population/year. The rates increased with age, and the age-specific rates were higher in women. To confirm the reduced survival of demented patients reported on the basis of individuals hospitalized at specific medical centers, we examined the survival of all demented individuals identified through our records-linkage study. Dealing with an entire population minimizes any possible selection bias that may be present for a series of patients seen at a particular medical institution. The survival rates generated for all demented patients in the defined population were significantly reduced compared to age- and sex-specific survival statistics derived from life-tables for residents of the northwest central region of the U.S., thereby documenting in a community study previous observations based on hospitalized patients.

The two-stage survey permitted us to estimate the prevalence of dementia in a biracial community. For each race, prevalence ratios were higher for females. For each race and sex, the prevalence figures rise dramatically with age. This morbidity study indicates that dementia represents a major health problem for both racial groups. All other studies are still in the data-collection phase.

Significance: Alzheimer's disease/senile dementia, despite its high apparent clinical frequency among the elderly, has not been well-studied in a U.S. population. These descriptive



studies are yielding etiologic hypotheses which can be further investigated using the case-control approach, and will provide the opportunity to investigate dementia occurring with other neurologic disorders, such as Parkinson's disease. These studies should also provide evidence for whether Alzheimer's disease occurring in the very elderly and that occurring in the presenium represent the same disease process.

Proposed Course of Project: Data collection will continue during the coming year. The two studies which have been completed have been presented at major national neurologic meetings and the results are being prepared for publication.

#### Publications:

Kokmen, E., and Schoenberg, B.S.: Epidemiological patterns and clinical features of dementia in a defined U.S. population. Trans. Am. Neurol. Assoc., in press.

Schoenberg, B.S.: Methodologic approaches to the epidemiologic study of dementia. In: Schuman, L.M., and Mortimer, J.A. (Eds.): Epidemiology of Dementia. London, Oxford University Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02241-05 ODIR
PERIOD COVERED <p style="text-align: center;">October 1, 1980 through September 30, 1981</p>		
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">The Epidemiology of Cerebrovascular Disease in Adults</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <div style="display: flex; justify-content: space-between; padding: 10px;"> <span>PI: Bruce S. Schoenberg      Chief</span> <span>NES      ODIR      NINCDS</span> </div>		
COOPERATING UNITS (if any) J.P. Whisnant, M.D., Mayo Clinic; D.G. Schoenberg, M.S., Bethesda, Maryland; A. Lilienfeld, Johns Hopkins University		
LAB/BRANCH Office of the Director, Intramural Research Program		
SECTION Neuroepidemiology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <p style="text-align: center;">1.3</p>	PROFESSIONAL: <p style="text-align: center;">1.3</p>	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS   <input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS         </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords)  <p>           This investigation is aimed (1) at evaluating the effect of <u>heart disease</u>            and <u>hypertension</u> as potentially treatable precursors of <u>completed stroke</u> and  <u>transient ischemic attacks</u>; (2) at documenting unusual patterns of            cerebrovascular disease; (3) at determining the <u>autopsy patterns</u> for patients            dying with cerebrovascular disease in a defined community; and (4) at            examining if <u>weather parameters</u> have any effect on stroke incidence.         </p>		

## Project Description:

Objectives: To determine the following: (1) the risk of stroke and transient ischemic attacks in individuals with heart disease and/or hypertension as compared to the risk in individuals without these conditions; (2) whether the existence of pre-existing heart disease and/or hypertension affects the type of stroke and whether it affects survival following stroke; and (3) whether there is a particular time interval following the onset of heart disease or hypertension during which an individual is at high risk for stroke. In addition, other studies address the issue of the effect of weather on stroke incidence, and a description of autopsy findings for patients dying with stroke in a defined community.

Methods Employed: This first study involves a non-concurrent prospective approach evaluating a cohort of 2,000 elderly individuals. The type of analysis follows the person-years strategy and utilizes life-table methods. The investigations of weather variables and autopsy patterns are based on the records-linkage resource for residents of Rochester, Minnesota.

Major Findings: When the case-control approach was applied to data available for the cohort of 2,000 individuals, different patterns of risk factors were demonstrated for TIA and completed ischemic stroke. While hypertension, diabetes mellitus, definite hypertensive heart disease, and valvular heart disease are important risk factors for completed stroke, these disorders have substantially less effect on the subsequent risk of TIA. When these data were analyzed in the format of a prospective study, it was possible to calculate the absolute risk of stroke as a function of the presence or absence of specific forms of cardiovascular disease. The following forms of cardiovascular disease yielded the highest ischemic stroke incidence rates (given in cases/1,000/year): myocardial infarction (15.5); congestive heart failure (20.5); and TIA (42.0). In considering risk factors for TIA, both angina/coronary insufficiency and congestive heart failure yielded the highest rates (10.4 and 10.9, respectively). Once etiologic precursors of stroke have been identified, medical intervention before the occurrence of long-lasting disability requires that there be an interval of time between the onset of the risk factor and the development of completed stroke. Analysis of data from this nonconcurrent prospective study revealed that those developing borderline hypertension, valvular heart disease, or ischemic heart disease remained stroke-free for the first 1-1/2 years after the first occurrence of each specific form of cardiovascular disease. This finding implies that there is an interval of time following the onset of these conditions when it may be possible to intervene medically to reduce the risk of stroke.

Other investigations in the area of stroke, involve the careful analysis of unusual patterns of cerebrovascular disease (e.g., more than 20 TIA's/day). The study of weather variables and stroke revealed that temperature has no effect on stroke incidence.

Significance: At the present time, there is little to suggest that improved medical management of the completed stroke will substantially affect the cerebrovascular disease problem. It would appear that greater benefit could be achieved by dealing with the precursors of stroke rather than delaying treatment until after the event has occurred. Previous studies of weather, showing a relationship to stroke mortality, were not confirmed when we examined incidence.

Proposed Course of Project: These results have been presented at international and national neurologic and epidemiologic meetings and the findings are being submitted for publication.

#### Publications:

Schoenberg, B.S.: The epidemiology of ischemic cerebrovascular disease. In Portera-Sanchez, A. (Ed.): Cerebral Ischemia. Geneva, Excerpta Medica, 1980, pp. 7-21.

Schoenberg, B.S.: Risk Factors for Cerebrovascular Disease. In Rose, F.C. (Ed.): Clinical Neuro-Epidemiology. Tunbridge, England, Pitman Medical, 1980, pp. 151-162.

Rosenblatt, K.A., Whisnant, J.P., and Schoenberg, B.S.: Temperature, snowfall, and the incidence of stroke: Rochester, Minnesota, 1955-1969. Stroke, in press.

Schoenberg, B.S., Schoenberg, D.G., Pritchard, D.A., Lilienfeld, A.M., and Whisnant, J.P.: Differential risk factors for completed stroke and transient ischemic attacks: study of vascular diseases (hypertension, cardiac disease, peripheral vascular disease) and diabetes mellitus. Trans. Am. Neurol. Assoc., in press.

Schoenberg, B.S.: Precursors of Stroke: Etiologic, Preventive, and Therapeutic Implications. New York, Oxford University Press, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02243-05 ODIR										
PERIOD COVERED <p style="text-align: center;">October 1, 1980 through September 30, 1981</p>												
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Pediatric Neuroepidemiology</p>												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Bruce S. Schoenberg</td> <td style="width: 33%;">Chief</td> <td style="width: 10%;">NES</td> <td style="width: 10%;">ODIR</td> <td style="width: 14%;">NINCDS</td> </tr> <tr> <td>Tatiana Kudrjavcev</td> <td>Neurologist</td> <td>NES</td> <td>ODIR</td> <td>NINCDS</td> </tr> </table>			PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS	Tatiana Kudrjavcev	Neurologist	NES	ODIR	NINCDS
PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS								
Tatiana Kudrjavcev	Neurologist	NES	ODIR	NINCDS								
COOPERATING UNITS (if any) D. Schoenberg, M.S., Research Epidemiologist, Bethesda, Maryland; J.F. Mellinger, M.D., and M.R. Gomez, M.D., Department of Neurology, Mayo Clinic; B.W. Christine, M.D., M.P.H., Connecticut State Department of Health												
LAB/BRANCH Office of the Director, Intramural Research Program												
SECTION Neuroepidemiology Section												
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205												
TOTAL MANYEARS: <p style="text-align: center;">2.5</p>	PROFESSIONAL: <p style="text-align: center;">2.5</p>	OTHER:										
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td colspan="3"><input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS</td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS						
<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER										
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS												
SUMMARY OF WORK (200 words or less - underline keywords)  <p>The project documented the frequency of <u>primary intracranial neoplasms</u> in the <u>pediatric populations</u> of Rochester, Minnesota, and the State of Connecticut. In addition, we investigated the magnitude and risk factors for <u>cerebrovascular disease in infants and children</u> in the Rochester, Minnesota population. Temporal trends in the incidence rate of <u>cerebral palsy</u> as well as distribution of clinical subtypes and survival by clinical subtype were determined for the population of Rochester, Minnesota, for the years 1950-1976.</p>												



## Project Description:

Objectives: To document 1) the frequency of primary neoplasms and cerebrovascular disease in pediatric populations; and 2) the temporal trends in the incidence of cerebral palsy. This had never been done before in a well-defined population group in the United States.

Methods Employed: Descriptive epidemiologic studies were carried out utilizing the resources of the Connecticut Tumor Registry and the Rochester, MN records-linkage system. In addition, a case-control study was undertaken to determine risk factors for perinatal intracranial hemorrhage.

Major Findings: The brain tumor incidence rate in children (<15 years of age) varied from 2.5-5.0 cases/100,000/year. The project demonstrated that neonatal intracranial hemorrhage is relatively common (1.1 cases/1,000 live births), that it is strongly associated with prematurity and hyaline membrane disease, and that it is difficult to recognize clinically. For pediatric cerebrovascular disease unassociated with birth, trauma, or infection, the incidence rate was 2.5/100,000/year. These cases were further characterized by survival, residual disability, and cause (whenever possible). The clinical and angiographic features of children with moyamoya disease were examined in detail. This condition appears to be more common than suggested by early case reports. With regard to cerebral palsy, our studies demonstrated that in recent years, incidence rates have declined. This trend has been especially marked for the spastic syndromes, with the exception of spastic diplegia which has remained relatively constant.

Significance: This study represents the first time that the magnitude of either brain tumors or cerebrovascular disease has been documented in a well-defined pediatric population.

Data from the study of cerebral palsy have been used to examine whether new developments in perinatal care affected the incidence of these conditions.

Proposed Course of Project: Additional diseases will be studied using similar methodology. A case-control study of cerebral palsy will follow the descriptive investigation. The problem of cerebrovascular disease in children will be re-evaluated following the introduction of computerized tomography on ultrasound as a diagnostic tool.



Publications:

Schoenberg, B.S., and Schoenberg, D.G.: The Spectrum of Pediatric Cerebrovascular Disease. In Rose, F.C., (Ed.): Clinical Neuro-Epidemiology. Tunbridge, England, Pitman Medical, 1980, pp. 319-329.

Schoenberg, B.S., (Ed.): Pediatric Neuroepidemiology. New York, Marcel Dekker, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> <b>INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 NS 02297-05 ODIR
PERIOD COVERED      October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Mortality from Neurologic Disorders: National and International Comparisons		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: Bruce S. Schoenberg      Chief      NES      ODIR      NINCDS		
COOPERATING UNITS (if any)  W. Massey, M.D., Duke University; D.G. Schoenberg, M.S., Bethesda, Maryland		
LAB/BRANCH Office of the Director, Intramural Research Program		
SECTION Neuroepidemiology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.7	PROFESSIONAL: 1.7	OTHER:
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  Although death certificate data are limited by possible misdiagnosis, incomplete case ascertainment, errors in coding, etc., detailed morbidity information on neurologic diseases for the entire U.S. and for other countries is not available. The Section has analyzed <u>mortality data for selected neurologic disorders</u> by country and by county in the U.S. The overall patterns which emerge may be useful in evaluating trends over time and in formulating etiologic hypotheses.		

Project Description:

Objectives: To analyze available mortality data by country and by county in the U.S.; to measure secular trends and formulate etiologic hypotheses.

Methods Employed: Age-adjusted death rates for selected neurologic disorders were calculated for 33 countries and for each U.S. county. Rates were ranked and patterns of mortality are being illustrated graphically and with maps.

Major Findings: Mortality from cerebrovascular disease has decreased in most developed countries over a 20-year period. This trend is not universal, however. For multiple sclerosis, countries initially reporting high mortality rates have generally reported declines while those with low rates earlier are reporting increases, so that the more recent mortality data for multiple sclerosis by country show less of a differential than previously observed.

Significance: Such detailed data are not available through sources of morbidity information. Consequently, we must utilize death certificate information to evaluate secular trends and patterns worldwide and by county within the U.S.

Proposed Course of Project: Analysis of these data is continuing, and publications will be prepared.

## PERIOD COVERED

October 1, 1980 through September 30, 1981

## TITLE OF PROJECT (80 characters or less)

Reviews of Epidemiologic Aspects of Neurologic Disease

## NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS
Tatiana Kudrjavcev	Neurologist	NES	ODIR	NINCDS

## COOPERATING UNITS (if any)

W. Massey, M.D., Duke University; D. Schoenberg, M.S., Bethesda, Maryland

## LAB/BRANCH

Office of the Director, Intramural Research Program

## SECTION

Neuroepidemiology Section

## INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Maryland 20205

## TOTAL MANYEARS:

2.0

## PROFESSIONAL:

2.0

## OTHER:

## CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS ☐ (b) HUMAN TISSUES ☒ (c) NEITHER☐ (a1) MINORS ☐ (a2) INTERVIEWS

## SUMMARY OF WORK (200 words or less - underline keywords)

Development of new neurologic studies requires thorough historic and methodologic reviews of prior investigations. These yield important unexplored etiologic clues that may be investigated using current technology. Major emphasis has been given to cerebrovascular disease, otitis media, inherited ataxias, Huntington's disease, febrile seizures, Tourette's syndrome, peripheral neuropathy, neurologic disease in the elderly, and controlled therapeutic trials of motor neuron disease.

## Project Description:

Objectives: To review comprehensively previous studies dealing with neurologic disorders in human populations, as well as other diseases with neurologic manifestations.

Methods Employed: Pertinent literature is critically reviewed for etiologic clues and unresolved issues which are answerable through properly designed neuroepidemiologic studies.

Major Findings: Suggested studies have been designed for cerebrovascular disease, otitis media, inherited ataxias, Huntington's disease, and febrile seizures. Some of these investigations are being pursued by the Section, as well as extramural scientists.

Significance: It is only through careful and critical review of previous efforts that a productive research program can be launched. These critical reviews are generally published so that both intramural and extramural investigators have access to this information.

Proposed Course of Project: These review efforts will continue and will generally focus on major neurologic diseases (e.g., dementia).

## Publications:

Schoenberg, B.S.: Neurologic disease in the elderly: Epidemiologic considerations. Seminars in Neurology 1: 5-12, 1981.

Kudrjavcev, T.: Differential Diagnosis and Work-up. In Nelson, K.B., and Ellenberg, J.H. (Eds.): NIH Consensus Development Conference on Febrile Seizures. New York, Raven Press, in press.

Schoenberg, B.S.: Controlled Therapeutic Trials in Motor Neuron Disease: Methodologic Considerations. In Rowland, L.P. (Ed.): Pathogenesis of Motor Neuron Disease. New York, Raven Press, in press.

Schoenberg, B.S.: Epidemiology of Peripheral Neuropathies. In Portera-Sanchez, A. (Ed.): Peripheral Neuropathies. Geneva, Excerpta Medica, in press.

Schoenberg, B.S.: Neuroepidemiologic Approach to Tourette Syndrome. In Friedhoff, A., and Chase, T. (Eds.): Tourette Syndrome. New York, Raven Press, in press.

Schoenberg, B.S.: Puzzling epidemics and neurochemical solutions: the interaction between neuroepidemiology and neurochemistry. In Rose, F.C. (Ed.): Metabolic Disorders of the Nervous System. Tunbridge, England, Pitman Medical, in press.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02300-05 ODIR										
PERIOD COVERED <p style="text-align: center;">October 1, 1980 through September 30, 1981</p>												
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Clinical Course and Medical Care for Neurologic Disorders</p>												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Bruce S. Schoenberg</td> <td style="width: 33%;">Chief</td> <td style="width: 10%;">NES</td> <td style="width: 10%;">ODIR</td> <td style="width: 14%;">NINCDS</td> </tr> <tr> <td>Other: F. Garcia-Pedroza</td> <td>Guest Worker</td> <td>NES</td> <td>ODIR</td> <td>NINCDS</td> </tr> </table>			PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS	Other: F. Garcia-Pedroza	Guest Worker	NES	ODIR	NINCDS
PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS								
Other: F. Garcia-Pedroza	Guest Worker	NES	ODIR	NINCDS								
COOPERATING UNITS (if any) <p style="text-align: center;">J.P. Whisnant, Dept. of Neurology, Mayo Clinic, Rochester, Minnesota</p>												
LAB/BRANCH <p style="text-align: center;">Office of the Director, Intramural Research Program</p>												
SECTION <p style="text-align: center;">Neuroepidemiology Section</p>												
INSTITUTE AND LOCATION <p style="text-align: center;">NINCDS, NIH, Bethesda, Maryland 20205</p>												
TOTAL MANYEARS: <p style="text-align: center;">2.0</p>	PROFESSIONAL: <p style="text-align: center;">2.0</p>	OTHER:										
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS   <input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS         </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div>												
SUMMARY OF WORK (200 words or less - underline keywords)  <p>         The study uses a review and abstraction of data from records for a selected group of <u>neurological disorders</u>. It obtains the items of data necessary to determine onset of the disorder, duration, date and cause of death, or current status. These data will be used to construct <u>modified life tables</u> to estimate the <u>expectation of life after diagnosis</u>, the survival curve and morbidity and severity estimates. It will also include analysis of type and duration of <u>medical care</u> received by patients with neurologic disorders derived from a <u>well-defined population</u>.       </p>												

Project Description:

Objectives: To determine the clinical course and patterns of medical care for all individuals with major neurologic disease in a given community.

Methods Employed: Cases of neurologic disease among residents of Rochester, Minnesota, have already been identified through previous studies. Medical encounters are documented through a records-linkage resource and this information has been abstracted.

Major Findings: The data for Rochester, Minnesota, residents with brain tumor or stroke have been collected and are being analyzed.

Significance: Rochester residents have access to high-quality medical care, and physicians with neurologic expertise are available within the community. Thus, the Rochester experience may provide some estimate of the pattern of medical care in the ideal situation in which the population has ready access to neurologic expertise, and in which there is little financial restraint to such care.

Proposed Course of Project: Data analysis and publication will follow.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 NS 02301-05 ODIR										
PERIOD COVERED <p style="text-align: center;">October 1, 1980 through September 30, 1981</p>												
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Collaborative Studies of Less Common or Less Debilitating Neurologic Disorders</p>												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Bruce S. Schoenberg</td> <td style="width: 33%;">Chief</td> <td style="width: 10%;">NES</td> <td style="width: 10%;">ODIR</td> <td style="width: 14%;">NINCDS</td> </tr> <tr> <td>Other: Tatiana Kudrjavcev</td> <td>Neurologist</td> <td>NES</td> <td>ODIR</td> <td>NINCDS</td> </tr> </table>			PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS	Other: Tatiana Kudrjavcev	Neurologist	NES	ODIR	NINCDS
PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS								
Other: Tatiana Kudrjavcev	Neurologist	NES	ODIR	NINCDS								
COOPERATING UNITS (if any) M. Zack, M.D., Atlanta, Georgia; Neurosciences Program, WHO, Geneva, Switzerland; D. Duane, M.D., B. Sandok, M.D., Mayo Clinic												
LAB/BRANCH Office of the Director, Intramural Research Program												
SECTION Neuroepidemiology Section												
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205												
TOTAL MANYEARS: <p style="text-align: center;">2.9</p>	PROFESSIONAL: <p style="text-align: center;">2.0</p>	OTHER: <p style="text-align: center;">0.9</p>										
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <span><input type="checkbox"/> (a) HUMAN SUBJECTS</span> <span><input type="checkbox"/> (b) HUMAN TISSUES</span> <span><input checked="" type="checkbox"/> (c) NEITHER</span> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <span><input type="checkbox"/> (a1) MINORS</span> <span><input type="checkbox"/> (a2) INTERVIEWS</span> </div>												
SUMMARY OF WORK (200 words or less - underline keywords)  <p>A number of collaborative efforts involve the investigation of the characteristics of unusual or less debilitating (e.g., headache) neurologic disease phenomena. Unusual associations or <u>space/time clusters of neurologic disorders</u> may provide leads to etiology or therapy. These may be tested <u>through more formal approaches.</u></p>												

## Project Description:

Objectives: To investigate and characterize unusual relationships, patterns, or phenomena associated with neurologic diseases.

Methods Employed: In collaboration with other governmental agencies (Center for Disease Control), international organizations (World Health Organization), and universities (Mayo Medical School) methods have been developed to investigate less common neurologic disorders. Several such studies have been completed. Current projects include investigation of space/time clusters of neurologic disease (with the Center for Disease Control), the development of survey strategies (with the World Health Organization and the Section on Disease Statistics Surveys), a study of myasthenia gravis and multiple sclerosis in the same patient (with the Mayo Clinic), and an investigation of neurologic disorders during pregnancy and the postpartum period (with the Mayo Clinic).

Major Findings: An unusual manifestation of aqueductal stenosis was reported. The magnitude of the Guillain-Barré syndrome was investigated in a well-defined population. This is only the second such study in the United States and provides a baseline for evaluating future phenomena (such as the increased occurrence with immunization against influenza A/New Jersey).

Proposed Course of Project: Several such ongoing studies will be analyzed and reported. New investigations are undertaken on an ad-hoc basis.

## Publications:

Hogg, J.E., Kobrin, D.E., and Schoenberg, B.S.: The Guillain-Barre syndrome - epidemiologic and clinical features. J. Chronic Dis. 32: 227-231, 1979.

Hogg, J.E., and Schoenberg, B.S.: Paralysis of divergence in an adult with aqueductal stenosis: case report. Arch. Neurol. 36: 511-512, 1979.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02305-05 ODIR										
PERIOD COVERED <p style="text-align: center;">October 1, 1980 through September 30, 1981</p>												
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">The Epidemiology of Intracranial Neoplasms</p>												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Bruce S. Schoenberg</td> <td style="width: 33%;">Chief</td> <td style="width: 10%;">NES</td> <td style="width: 10%;">ODIR</td> <td style="width: 14%;">NINCDS</td> </tr> <tr> <td>Other: Tatiana Kudrjavcev</td> <td>Neurologist</td> <td>NES</td> <td>ODIR</td> <td>NINCDS</td> </tr> </table>			PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS	Other: Tatiana Kudrjavcev	Neurologist	NES	ODIR	NINCDS
PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS								
Other: Tatiana Kudrjavcev	Neurologist	NES	ODIR	NINCDS								
COOPERATING UNITS (if any)      B.W. Christine, M.D., M.P.H., Connecticut State Dept. of Health; J.P. Whisnant, M.D., and R.J. Campbell, M.D., Mayo Clinic; L. Mahalak, M.D., Jackson, MS; A. Heck, M.D., Univ. of TN; R. Simon, M.D., Berkeley, CA; B. Jordan, B.A., Harvard Medical School												
LAB/BRANCH Office of the Director, Intramural Research Program												
SECTION Neuroepidemiology Section												
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205												
TOTAL MANYEARS: <p style="text-align: center;">1.3</p>	PROFESSIONAL: <p style="text-align: center;">1.3</p>	OTHER:										
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;"> <input type="checkbox"/> (a) HUMAN SUBJECTS         </div> <div style="width: 30%;"> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div> <div style="display: flex; justify-content: space-between; align-items: flex-start; margin-top: 5px;"> <div style="width: 30%;"> <input type="checkbox"/> (a1) MINORS         </div> <div style="width: 30%;"> <input type="checkbox"/> (a2) INTERVIEWS         </div> </div>												
SUMMARY OF WORK (200 words or less - underline keywords)  <p>The Section has conducted extensive investigations on the descriptive epidemiology of primary intracranial neoplasms using data derived from population-based registries worldwide. Analytic studies were carried out to investigate the relationship between intracranial neoplasms and tumors occurring at other sites. These studies included careful review of tumor nomenclature, disease definitions, and survey strategies.</p>												

## Project Description:

Objectives: To resolve problems in nomenclature, disease definitions, and survey strategies; to define the magnitude and distribution of primary intracranial neoplasms; and to investigate the relationship between intra- and extracranial tumors.

Methods Employed: Descriptive epidemiologic techniques were applied to data obtained from tumor registries around the world. Wherever possible, cases were reviewed by the Section's staff. New analytic techniques for studies of multiple primary cancers were devised.

Major Findings: On the basis of the descriptive studies, two patterns of age-specific incidence emerged. Analyses of most population-based data worldwide revealed a small childhood peak, followed by a later peak between ages 50 and 80. Data for Rochester, Minnesota, however, showed the childhood peak, followed by an increasing incidence rate with increasing age. Careful study of this discrepancy showed 1) that the greater percentage of cases first diagnosed at autopsy in Rochester accounted in large part for this difference, and 2) that a substantial number of brain tumors remain undiagnosed in the elderly during life. Studies are currently underway to evaluate the role of computerized tomography in the diagnosis of brain tumors and to explain the recent increase in the incidence of pituitary tumors among women of child-bearing age. In addition, racial differentials in the frequency of certain intracranial tumors (meningiomas and pituitary adenomas) are being examined. Investigations of the relationship between intracranial neoplasms and extracranial tumors have been especially rewarding. An association was found between the occurrence of breast cancer and meningioma in women. This result raises interesting etiologic possibilities when considered with other evidence: 1) meningioma is the only common intracranial neoplasm with a higher incidence in females; 2) the abrupt clinical appearance or enlargement of this tumor during pregnancy has been described; and 3) the finding of estrogen receptor protein in meningioma has been reported. This has led to further work concerning estrogen receptors in other tumors such as malignant melanoma involving the nervous system.

Significance: Previous epidemiologic studies regarded all brain tumors as a single disease. The set of studies described above provided overwhelming evidence that the individual histologic types of primary intracranial neoplasms represent distinct disease entities. The analytic studies for the first time began to define risk factors for these tumors. The possible role of hormones in the etiology or growth of meningiomas may have therapeutic significance.



Proposed Course of Project: Several of these findings are being prepared for publication.

Publications:

Annegers, J.A., Schoenberg, B.S., Okazaki, H., and Kurland, L.T.: Intracranial Neoplasms in Rochester, Minnesota, 1935-1977. In Rose, F.C., (Ed.): Clinical Neuro-Epidemiology. Tunbridge, England, Pitman Medical, 1980, pp. 366-371.

Schoenberg, B.S., and Christine, B.W.: Malignant melanoma associated with breast cancer. South. Med. J. 73: 1493-1497, 1980.

Annegers, J.F., Schoenberg, B.S., Okazaki, H., and Kurland, L.T.: Epidemiologic study of primary intracranial neoplasms. Arch. Neurol. 38: 217-219, 1981.

Kurland, L.T., Schoenberg, B.S., Annegers, J.F., Okazaki, H., and Molgaard, C.A.: The incidence of primary intracranial neoplasms in Rochester, Minnesota, 1935-1977. Accepted for publication in the Annals of the New York Academy of Sciences.

Schoenberg, B.S.: Cancer of Specific Tissues: Nervous System. In Schottenfeld, D., and Fraumeni Jr., J.F. (Eds.): Cancer Epidemiology and Prevention. Philadelphia, W.B. Saunders, in press.

Schoenberg, B.S.: Epidemiology of Brain Tumors. In Walker, M.D. (Ed.): Oncology of the Nervous System. Hingham, Massachusetts, Martinus Nijhoff, in press.



## Project Description:

Objectives: The severe shortage of available manpower in neuroepidemiology necessitated development of an educational program which was initiated by the Section.

Methods Employed: A series of four videotapes were produced by the Section and are distributed on a loan-basis without charge - the 1977 course and conference were held in cooperation with Georgetown University. The 1979 Symposium was held in conjunction with Yale University. A textbook on neurological epidemiology was published by Raven Press in 1978.

Major Findings: Attendance at the conferences included over 250 representatives from Asia, Africa, Europe, Latin America, and the U.S. Approximately 2,000 copies of the textbook have been requested. The Section in cooperation with the World Health Organization, organized the following courses in neuroepidemiology: a three-day course was held in Beijing, People's Republic of China in 1980; a one-week course took place in Madrid, Spain in 1981; and an advanced international course was carried out in Florence, Italy in 1981. There were 100 professors of neurology and neurosurgery that attended the China course, there were approximately 60 registrants (neurologists) from Spain and the Canary Islands at the Madrid course, while the Florence course attracted 250 registrants (only 130 could be accommodated). Although most attending the Florence symposium were Italian, other participants were from Korea, Australia, the Philippines, Canada, the United States, Ecuador, Peru, England, France, Spain, Greece, Germany, Norway, Sweden, Finland, Poland, Czechoslovakia, Israel, and Nigeria. In addition, there will be a three-day symposium in conjunction with the meeting of the International Epidemiological Association in Europe during 1981 and a one-day symposium in conjunction with the World Congress of Neurology during 1981 in Kyoto, Japan. A one-day course is planned for 1982 in conjunction with the American Academy of Neurology.

Significance: With such a limited supply of expertise in neurological epidemiology, these educational resources fill an important need in the neurosciences. Such activities provide a stimulus for the development of research activities in other countries. Research units have been organized in Mexico, India, Spain, Nigeria, and the People's Republic of China.

Proposed Course of the Project: Further courses and additional videotapes have been requested. Furthermore, videotapes are being converted for use in Europe and Asia.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02370-03 ODIR										
PERIOD COVERED <p style="text-align: center;">October 1, 1980 through September 30, 1981</p>												
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Racial Differentials in the Prevalence of Major Neurologic Disorders</p>												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Bruce S. Schoenberg</td> <td style="width: 33%;">Chief</td> <td style="width: 10%;">NES</td> <td style="width: 10%;">ODIR</td> <td style="width: 14%;">NINCDS</td> </tr> <tr> <td>Dallas Anderson</td> <td>Survey Statistician</td> <td>OBFS</td> <td>OD</td> <td>NINCDS</td> </tr> </table>			PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS	Dallas Anderson	Survey Statistician	OBFS	OD	NINCDS
PI: Bruce S. Schoenberg	Chief	NES	ODIR	NINCDS								
Dallas Anderson	Survey Statistician	OBFS	OD	NINCDS								
COOPERATING UNITS (if any) Office of Biometry and Field Studies, OD, NINCDS; A. Haerer, M.D., University of Mississippi; U.S. Bureau of the Census												
LAB/BRANCH Office of the Director, Intramural Research Program												
SECTION Neuroepidemiology Section												
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205												
TOTAL MANYEARS: 7.5	PROFESSIONAL: 4.5	OTHER: 3.0										
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER												
<input checked="" type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS												
SUMMARY OF WORK (200 words or less - underline keywords)  <p>The purpose of this study is to accurately document possible <u>racial</u> differentials in the prevalence of major <u>neurologic disorders</u> by surveying an entire county, with a biracial population of approximately 25,000. The disorders investigated include <u>cerebral palsy</u>, <u>dementia</u>, <u>psychomotor delay</u>, <u>epilepsy</u>, <u>Parkinson's disease</u>, <u>essential tremor</u>, and <u>cerebrovascular disease</u>.</p>												

Project Description:

Objectives: To accurately document possible racial differentials in the prevalence of major neurologic disorders in a well-defined biracial population.

Methods Employed: A strategy was developed which eliminated the requirement that persons must have entered the health care system for detection of disease. The basis of the investigation was a door-to-door survey which utilized a detailed questionnaire inquiring not only about diagnoses, but also about signs and symptoms suggestive of neurologic dysfunction. Over 99% of the households agreed to the interview. Those household members suspected of having one of the disorders of interest were then asked to have a neurologic examination conducted by a senior, board-certified neurologist.

Major Findings: Data currently available for Parkinson's disease indicate that in the population studied, parkinsonism is more common in whites, but the difference between races is not as great as suggested by earlier studies. The same survey yielded information on essential tremor, thereby providing the first data on the prevalence of this condition in a defined U.S. population. For either race, the prevalence ratios were slightly greater in women, and for either sex the figures were slightly higher for whites.

Significance: A number of early investigations suggested possible differences in neurologic disease frequency by race, but were based on hospital or clinic experience and these studies could not identify a well-defined population from which cases were derived. Population-based studies followed, but questions concerning the results centered on possible racial differentials in access to expertise in neurologic diagnosis and treatment. The present study should eliminate these potential sources of bias.

Proposed Course of Project: The interviews and examinations have been completed, and the data are being edited and analyzed. Similar strategies are being developed for application in developing countries, in collaboration with the World Health Organization.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02423-02 ODIR
PERIOD COVERED <p style="text-align: center;">October 1, 1980 through September 30, 1981</p>		
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Development of Data Resources for Neuroepidemiology</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <p>             PI: Bruce S. Schoenberg    Chief                      NES    ODIR    NINCDS           </p>		
COOPERATING UNITS (if any)    F. Clifford Rose, M.B., F.R.C.P., B. Benjamin, Ph.D., S. Haberman, M.A., F.I.A., and R. Capildeo, M.B., B.S., Charing Cross Neuroepidemiology Unit, London, England; W. Sibley, M.D., Univ. of Arizona, Tucson, Arizona; and R. Katzman, M.D., Albert Einstein Sch. of Med., N.Y., N.Y.		
LAB/BRANCH <p style="text-align: center;">Office of the Director, Intramural Research Program</p>		
SECTION <p style="text-align: center;">Neuroepidemiology Section</p>		
INSTITUTE AND LOCATION <p style="text-align: center;">NINCDS, NIH, Bethesda, Maryland 20205</p>		
TOTAL MANYEARS: <p style="text-align: center;">1.1</p>	PROFESSIONAL: <p style="text-align: center;">1.1</p>	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS         </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div> <div style="margin-top: 5px;"> <input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS       </div>		
SUMMARY OF WORK (200 words or less - underline keywords)  <p>       To develop 1) a <u>registry of hospitalized patients with neurologic diseases</u> in        a well-defined population of 3.5 million people, and 2) <u>resources for</u>  <u>case-control studies of multiple sclerosis and Alzheimer's disease</u> using  <u>uniform methods of data collection.</u> </p>		



## Project Description:

Objectives: To develop a functioning registry of hospitalized patients with neurologic disease in a well-defined population of 3.5 million inhabitants. The project also involves the establishment of resources for case-control studies of multiple sclerosis and Alzheimer's disease, utilizing a uniform data base for each disorder.

Methods Employed: Based on information routinely collected by the British National Health Service, a registry was established for all neurologic inpatients derived from a population of 3.5 million (North-West Thames Metropolitan Region, London, England). Recorded data include demographic information, length of hospitalization, details of surgical procedures, and up to five diagnoses. The registry data are being tested for accuracy of diagnosis and coding, hospital and physician cooperation, completeness of case ascertainment, and delay from hospital discharge to data entry in the registry. The Registry's operation was investigated with a study of the Guillain-Barré syndrome.

For the establishment of resources for case-control studies, a uniform data base is being established for collaborative studies of multiple sclerosis and Alzheimer's disease.

Major Findings: The initial numbers of cases of the Guillain-Barré syndrome are in line with what would be expected on the basis of U.S. statistics.

Significance: An important problem for the neuroepidemiologist is the enormous cost of maintaining neurologic surveillance on a large number of patients and the long period of time generally required to investigate temporal patterns. The establishment of a registry of neurologic diseases based on an ongoing government-supported collection of data, will allow neuroepidemiologists to respond rapidly to etiologic questions at minimal cost. The use of a uniform data base for case-control studies will permit the pooling of information from several centers, thereby facilitating the examination of relatively uncommon phenomena.

Proposed Course of Project: Attempts will be made to improve the efficiency of the Registry's operation.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02424-02 ODIR
PERIOD COVERED <p style="text-align: center;">October 1, 1980 through September 30, 1981</p>		
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Standardized Nomenclature and Coding of Neurologic Diseases</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <div style="display: flex; justify-content: space-between; padding: 10px;"> <span>PI: Bruce S. Schoenberg    Chief</span> <span>NES    ODIR    NINCDS</span> </div>		
COOPERATING UNITS (if any) L. Kurland, M.D., Mayo Clinic, Rochester, MN; J.F. Kurtzke, M.D., Georgetown Univ., Washington, D.C.; F. Clifford Rose, M.B., F.R.C.P.; B. Benjamin, Ph.D., S. Haberman, M.A., F.I.A., and R. Capildeo, M.B., B.S.; Charing Cross Neuroepidemiology Unit, London, England; L. Schut, M.D., Minneapolis, MN; and K. Kondo, Niigata, Japan		
LAB/BRANCH <p style="text-align: center;">Office of the Director, Intramural Research Program</p>		
SECTION <p style="text-align: center;">Neuroepidemiology Section</p>		
INSTITUTE AND LOCATION <p style="text-align: center;">NINCDS, NIH, Bethesda, Maryland 20205</p>		
TOTAL MANYEARS: <p style="text-align: center;">1.1</p>	PROFESSIONAL: <p style="text-align: center;">1.1</p>	OTHER:
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS   <input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS         </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords)  <p style="text-align: center;">To develop an internationally acceptable <u>standard</u> of <u>nomenclature</u>, <u>classification</u>, and <u>coding</u> of <u>neurologic disorders</u>.</p>		

Project Description:

Objectives: To develop an internationally acceptable standard of nomenclature, classification, and coding of neurologic disorders.

Methods Employed: The new system of classification and coding first follows a thorough review of existing schemes, with particular attention to deficiencies. The resulting new system will attempt to correct these deficiencies and aim to facilitate compliance by physicians.

Major Findings: A review of the 9th revision of the International Classification of Diseases reveals that it is seriously deficient with regard to neurologic diseases. Disorders with different etiologies and different epidemiologic patterns are categorized together. Neurologic diseases may be classified under endocrine or psychiatric conditions.

Significance: Epidemiologic studies have two basic requirements: uniformity and accuracy of data collection. This necessitates the use of a standardized, internationally acceptable classification and coding scheme that can be easily used by physicians, regardless of the available medical facilities.

Proposed Course of Project: Pilot studies will be carried out to test and revise the new classification and coding scheme. This will be followed by periodic meetings to consider the adaptation of this system. The final results will be presented to the World Health Organization for possible incorporation into their own International Classification of Diseases.





## ANNUAL REPORT

October 1, 1980 through September 30, 1981

### Neurotoxicology Section, ODIR

National Institute of Neurological and Communicative Disorders and Stroke

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Annual Report  
for period October 1, 1980 through September 30, 1981  
Neurotoxicology Section  
Office of the Director  
Intramural Research Program  
National Institute of Neurological and Communicative  
Disorders and Stroke  
Ellen K. Silbergeld, Chief

SUMMARY

1. Neurochemical Bases of Lead Neurotoxicity in Children

Low level lead exposure of young children is associated with increased release of the neurotransmitter dopamine, among other neurochemical changes. Dopaminergic effects of lead provide a clinical outcome which is measurable noninvasively, that is, the excretion of the metabolite homovanillic acid (HVA) in urine. Increased urinary HVA was observed in over 50 lead-exposed children studied, and was found to be positively and significantly correlated with elevations in blood lead above 30 ug/100 ml. There were no correlations between increases in urinary HVA and heme precursors, suggesting that this widely used measure of peripherally mediated lead toxicity does not accurately predict CNS toxicity. In addition, a study of approximately 24 children exposed more than once to lead showed greater increases in HVA elevation than children exposed for the first time, although their degree of absorption was approximately the same. Thus, although the dopaminergic effects of lead may be reversible by cessation of lead exposure and by chelation therapy, a prior history of lead exposure potentiates the neurochemical effects of subsequent, low level exposure. The results point to the need to prevent exposure to lead as the best method to prevent neurotoxicity.

2. Neurotoxic Aspects of Porphyrinopathies

Several metals, including lead, as well as pesticides and other polyhalogenated hydrocarbons, affect the biosynthesis of heme in cells. Because of similarities among these intoxications (particularly in neurotoxic signs) and hereditary disorders of heme synthesis, the porphyrias, it has been hypothesized that altered heme synthesis may in itself have neurotoxic sequelae. We have shown that the GABAergic effects of lead may involve the effects of accumulation of a heme precursor toxic to GABAergic synapses, rather than a direct action of lead itself at these sites. Succinyl acetone has been identified as a potent and specific inhibitor of the rate-limiting enzyme in the biosynthesis of heme. Administration of succinyl acetone for periods as short as one week produces significant inhibition of GABAergic neurotransmission. These results are the first demonstration of heme-related neurotoxicity using a specific inhibitor of heme synthesis. In addition, the results of these studies may be of interest to experimental therapeutics since succinyl acetone can inhibit growth of certain tumors in vitro and in vivo. Possible neurotoxicity of this drug requires further study before contemplating its use in cancer therapy.

3. Neurotoxicity of Erythrosin B

Erythrosin B (Tetraiodofluorescein, U.S.F.D. & C. RED No. 3) is an artificial food and drug color of the xanthene or fluorescein family. We reported earlier that in vitro it can block synaptosomal uptake of DOPamine. We have now demonstrated that erythrosin and three of its structural analogs

are highly potent and specific inhibitors of the Na,K-ATPase unique to brain tissue, and in brain, to tissue other than purified myelin. [14C]-erythrosin B appears to interact with brain synaptic membranes in a saturable, "receptor-like" manner at a site closely linked to, but not identical to, the glycoside binding site of the ATPase. Studies on the behavioral neurotoxicity of erythrosin B have some behavioral effects some of which are strain-dependent. There is also potent behavioral toxicity following direct administration of 400 nanomoles/10  $\mu$ l amounts of dye into the lateral ventricles. These effects are comparable to the ICV effects of ouabain, whose neurotoxicity is also poorly understood. There are significant discrepancies between these acute *in vivo* behavioral studies and the acute *in vitro* studies of brain ATPase, which suggest that the biochemical studies undertaken to date may not completely explain any neurobehavioral toxicity associated with xanthene dyes. Another aspect of the cellular toxicity of erythrosin B and some of its structural analogs is its ability to inhibit the growth of neurites of chick dorsal root ganglia in culture. This effect may involve a photo-oxidative effect of erythrosin on nerve growth factor; however, other, light-insensitive actions of erythrosin on both neurons and fibroblasts were also observed in these studies.

In collaboration with the department of biochemistry, University of Miami Medical School, we have studied another ATPase which appears sensitive to erythrosin, the CA-transporting, Ca,mg-supported ATPase of purified sarcoplasmic reticulum. Both its CA-transporting ability and catalytic activity are sensitive to erythrosin B in the micromolar range. These effects are light-insensitive and distinct from any glycoside-like effect since this tissue contains no enzyme that is sensitive to ouabain, nor specific glycoside binding sites.

#### 4. Hormones and Central Neurotransmitter Function

Previous studies showed that administration of the gonadal hormone estradiol produces an increased number of dopamine receptors in the striatum of male rats and associated evidence of behavioral supersensitivity of these receptors to both agonists (amphetamine and apomorphine) and antagonists (haloperidol) of dopaminergic function. Using multiple behavioral measures, we have shown that estrogen potentiates haloperidol-induced catalepsy. This finding raises the question as to whether estrogen modulates DA-mediated behaviors through striatal postsynaptic DA receptor supersensitivity alone.

Since the effects of estrogen can be prevented by hypophysectomy, we have tested the activity of the pituitary hormone prolactin, whose release is augmented by estradiol. Prolactin also increases density of striatal dopamine receptors in male rats. Female and male rats have different responses to manipulations of hormone function and different interrelationships between numbers of striatal dopamine receptors and behaviors thought to be mediated by these receptors. Intact female rats normally display more intense stereotyped behavior than males, although there are no differences in number of sprioperidol receptors in male and female striatal tissue. While estradiol produces similar biochemical evidence of increased dopaminergic function (increased density of sprioperidol binding sites), the behavioral effects of estradiol are very complex and depend upon the state of the animal.

#### 5. Studies of Genetic Variation in Response to Neurotoxins and CNS Function

Two aspects of genetically determined variation in neurochemistry have been studied with the hypothesis of correlating differences in toxin reponse. Liver and brain monoamine oxidase activities (type A and B) were

measured in 20 inbred rat strains. Substantial variation was observed among these strains in rates of phenethylamine, tyramine, and 5-HT oxidation. The observed low correspondence between rates of phenylethylamine and 5-HT oxidation may reflect structural or modulatory differences in MAO A and B activities.

NA,K-ATPase activity was measured in rat cortices from 12 inbred strains. Two-fold variation was found between the strains with the highest and lowest ATPase activity. Genetically heterogeneous NIH rats were examined between the ages of 11 and 90 days for differences in [ $^3$ H]-ouabain binding. Two binding sites were found for all ages except the 11 day old rats, where only the high affinity site could be demonstrated. This may be related to myelination, since other studies suggest that the low affinity site is located in the myelin fraction of rat brain.

#### 6. Chromaffin Granules and Chromaffin Cells

The chromaffin cell provides a well-studied system for investigating molecular and cell-surface mediated mechanisms of neurotoxin action. Since several neurotoxins of interest to neurology are divalent cations (lead, manganese, copper) and since storage granules, such as chromaffin granules, synaptic vesicles, and platelet granules contain high concentrations of calcium, these preparations have been investigated to determine the effect of toxic cations on calcium storage and calcium-mediated processes of fusion and exocytosis.

X-ray microanalysis and X-ray excited fluorescence studies demonstrate that in platelet granules the high concentrations of calcium are stored in a crystalline array; studies are continuing on other particles, and on the effects of other cations on this calcium storage matrix.

Chromaffin granules will aggregate and fuse in the presence of calcium. This reaction is independent of ATP and is not inhibited by a phosphodiesterase inhibitor, theophylline. Rapid freeze fracture electron microscopic studies demonstrate that membrane-associated particles move prior to fusion. Aggregation studies by light scattering readout from a stopped-flow apparatus have been extended using fluorescent energy transfer. Results have provided the first demonstration of the fluid mosaic structure of the membrane of a subcellular organelle. Granule-granule recognition and aggregation is mediated by protruding proteins; however, labelling studies indicate that these proteins contain no free sulfhydryls or that no significant detectable energy transfer occurs because of the geometry of these particles.

#### 7. Drug-Induced Immobility States and Animal Analogs of Parkinsons Disease

To elucidate the neural substrates underlying different forms of drug-induced immobility states, in particular, neuroleptic and opiate catalepsy, as animal (rat) analogs of Parkinson's disease, we have studied the contribution of vestibular mechanisms to neuroleptic-induced bracing reactions, an animal analog of Parkinsonian freezing reactions. A new technique of eighth nerve deafferentation has been developed for behavioral studies on the effects of unilateral and bilateral vestibular damage on bracing, grasping, righting and ocular reactions before and after treatment with haloperidol. These experiments are also relevant as experimental analogs of the clinical use of neuroleptics in human vestibular disorders.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02319-04 ODIR				
PERIOD COVERED October 1, 1980 to September 30, 1981						
TITLE OF PROJECT (80 characters or less)  Analytic Electron Microscopy in Neurochemistry						
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%;"> <tr> <td style="width: 50%;">PI: Ellen K. Silbergeld, Chief</td> <td style="width: 50%;">NTS NINCDS</td> </tr> <tr> <td>Other: C. Fiori</td> <td>Physicist BEIB NIH</td> </tr> </table>			PI: Ellen K. Silbergeld, Chief	NTS NINCDS	Other: C. Fiori	Physicist BEIB NIH
PI: Ellen K. Silbergeld, Chief	NTS NINCDS					
Other: C. Fiori	Physicist BEIB NIH					
COOPERATING UNITS (if any) Department of Neuropathology, Johns Hopkins Hospital, Baltimore MD; Department of Neurology, Tufts Medical School, Boston MA; Department of Neurology, Univ. of Michigan Medical School, Ann Arbor, MI; BEIB, NIH						
LAB/BRANCH Office of the Director, Intramural Research Program						
SECTION Neurotoxicology Section						
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland						
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TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.3	OTHER: 0.2				
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS						
SUMMARY OF WORK (200 words or less - underline keywords)  Recent technological advances in coupling electron microscopy to analytic techniques involving electron-electron interactions have been applied to neurochemistry and neuropathology. Localization of heavy metals was successfully studied at the subcellular level in experimental lead poisoning, <u>cis-platinum treatment</u> , and <u>manganese exposure</u> . Clinically, <u>brain tissue</u> from patients with <u>Alzheimers disease</u> and <u>amyelotrophic lateral sclerosis</u> was studied for increased local concentrations of aluminum, lead, and other toxic metals.						



## Project Description

### Objectives:

- (1) to determine the applicability of new techniques in analytic electron microscopy (x-ray microprobe analysis and electron energy loss spectroscopy) to studying neurochemistry and neurotoxicology.
- (2) to determine local concentrations of toxic metals in brain tissue in relation to possible sites and mechanisms of action in the CNS.

### Methods employed:

Analytic electron microscopy exploits electron-electron events occurring when specimens containing elements and molecules are bombarded with the x-ray beam used to produce images in scanning-transmission or transmission-electron microscopy (STEM or TEM). Using new instrumentation developed at the Biomedical Engineering and Instrumentation Branch, NIH, both clinical and experimental tissues have been examined. A critical problem in such studies is tissue preparation, since at least two factors can interfere with analysis. One is the addition of exogenous compounds during tissue preparation for conventional electron microscopy (osmium, lead, and uranyl compounds, used in staining and fixation); the other is the loss of endogenous compounds during fixation, embedding and analysis (particularly diffusible ions). Several types of preparations are used to compare the impact of these two factors: rapidly air-dried homogenates and purified fractions of subcellular components; and variations of conventional and freeze-substituted preparations of thin-sectioned material. In collaboration with the Department of Neuropathology, Johns Hopkins, and the Department of Neurology, Tufts Medical School, we have examined conventionally prepared material from human autopsy representing several diseases in which abnormal metabolism of metals has been hypothesized to play a part, either as etiology or pathology. In addition, considerable work has been done in collaboration with BEIB on preparing standards for quantitative analysis and determining limits of detection.

In a continuing project, tissue was examined from patients with Alzheimer's disease and amyotrophic lateral sclerosis (ALS), in collaboration with the Department of Neurology, Tufts Medical School. Some tissue appeared to contain high levels of aluminum in myelinated fibers of the spinal cord (Alzheimer's); however, problems of elemental contamination may compromise these results. No notable subcellular concentrations of lead were found in ALS specimens, but these samples are being reanalyzed under different conditions.

### Proposed course

Further studies are planned for examination of postmortem tissue from several neurological disorders in which increased local concentrations of metals is hypothesized to occur.

Publications:           None.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  201 NS 02264-05 ODIR
PERIOD COVERED    October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Animal Models of Neurological Disease		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:  Other:	Ellen K. Silbergeld Robert E. Hruska Sally M. Anderson Marc De Ryck Stephen J. Morris Hugh A. Tilson Donald Tschudy J. Julian Chisolm David Thomas Duncan Haynes D.W. Albers Diane Bradley Robin Greenler	Chief Staff Fellow Expert Consultant Visiting Fellow Expert Consultant Pharmacologist Medical Officer Johns Hopkins School of Medicine Baltimore City Hospitals University of Miami Medical School Chief, ECS Biological Aid Biological Aid  NTS    NINCDS NTS    NINCDS NTS    NINCDS NTS    NINCDS NTS    NINCDS LNBT    NIEHS MET    NCI  LNC    NINCDS NTS    NINCDS NTS    NINCDS
COOPERATING UNITS (if any)    Department of Pediatrics, Johns Hopkins School of Medicine, Baltimore, MD; Division of Hematology Research, Scripps Clinic, La Jolla, CA; LNBT, NIEHS; MET, NCI		
LAB/BRANCH Office of the Director, Intramural Research Program		
SECTION Neurotoxicology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:  3.8	PROFESSIONAL:  2.7	OTHER:  1.1
CHECK APPROPRIATE BOX(ES)		
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<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) This project has concerned the development of animal models of neurological disease produced by exposure to synthetic and naturally occurring <u>neurotoxins</u> in the environment. The interaction of various toxins and <u>neurotransmitters</u> and hormones in the CNS have provided the focus for combined <u>behavioral and neurochemical</u> studies emphasizing basic mechanisms of action of proposed <u>neurotoxins</u> . Major topics studied this past year were: (1) effects of <u>heavy metals</u> on neurotransmitter and neuroendocrine function; (2) <u>neurotoxic mechanisms</u> of chemical and hereditary porphyriopathies; (3) effects of <u>artificial food colors</u> on neuronal membranes and neurotransmission; (4) role of <u>vestibular</u> function in posture and motor function.		

## Project Description

### Objectives:

- (1) to develop animal models of neurological disease caused by environmental neurotoxins and other compounds;
- (2) to explicate the mechanisms of action of these neurotoxins at specific sites in the central nervous system;
- (3) to study neurotoxic disease in clinical populations; and
- (4) to investigate the genetic basis for response to neurotoxins.

### A. Neurochemical bases of lead neurotoxicity

Methods employed: Experimentally, chronic and acute in vivo exposure paradigms are used to study the effects of lead on central nervous system biochemistry and correlated behaviors in rats. Neurochemical studies have concentrated upon GABAergic and dopaminergic interactions with a view to understanding specific behavioral symptoms of hyperactivity, hyperreactivity, and convulsions. Clinically, a population of lead-exposed children has been studied through the Lead Poisoning Clinics of Johns Hopkins Medical School Department of Pediatrics and the Baltimore City Hospitals.

Major findings etc: As demonstrated previously in this project, low level lead exposure of immature rodents is associated with significant increases in synaptic release of the transmitter dopamine, primarily from limbic system pathways. These effects on dopaminergic neurotransmission provide a measurable outcome, detectable by noninvasive techniques, in human populations, - that is, measurement of dopamine metabolites in quantitative 24-hr urine collections made under controlled clinical conditions. In rats and mice, increases in urinary dopamine metabolites (homovanillic acid and dihydroxyphenylacetic acid) can be correlated with increased levels of these metabolites in brain and cerebrospinal fluid; in children, it may be inferred that increased CNS dopaminergic neurotransmission is at least in part involved in the observed increases in urinary metabolites. However, other factors may also be involved, including lead-induced changes in blood-CSF transfer, altered patterns of catabolism, defects in renal function, and overactivity of peripheral catecholaminergic function.

The clinical studies reported previously have been continued in collaboration with Dr. J. Julian Chisolm of Johns Hopkins, and will be reported on at the third International Conference on Heavy Metals in the Environment in September 1981. Consistent with earlier studies, significantly increased homovanillic acid (HVA) (more than 2 standard deviations above the normal range) was found in children with elevations in blood lead above 30 ug/100 ml. This is the level of lead absorption which has been associated, in neuropsychiatric studies, with significant decrements in learning and fine motor performance. In most children, increased HVA was reversed after longterm therapy with chelating drugs; coadministration of EDTA and BAL produced a more rapid decline in HVA than treatment with EDTA alone. Children exposed more than once to elevated lead burdens (which occurs when they return to leaded environments after release from hospital) show greater increases in HVA than children exposed for the first time, given equal absorption of lead (measured by blood lead concentration). These preliminary observations are of great clinical importance, indicating that the best method for avoiding neurotoxic sequelae of lead poisoning is prevention of exposure. The apparent potentiation of the effects of lead by prior exposure is consistent with experimental studies in rodents which have shown that prior exposure to lead

increases the uptake and retention of subsequent doses by the central nervous system.

Attempts were made, on the basis of preliminary experimental work reported on last year, to determine if any plasma hormone measurements might provide other sensitive indicators of low level lead neuroendocrine toxicity not requiring hospitalization to ensure quantitative 24-hour sample collections. Animal experiments had suggested that lead exposure can alter plasma prolactin and growth hormone levels. However, normally these hormones circulate in very low levels in the young child and little is known of the normal range. Also, plasma hormones are quite labile, rising rapidly in response to stress, trauma, and activity. These factors are difficult to control in pediatric populations. The variability of levels found in 10 children studied indicate that plasma hormones are unlikely to provide reliable indicators of response of the hypothalamus and/or pituitary to lead exposure, and this part of the project has been terminated.

Projected Course: Clinical studies on lead will continue in order to define more conclusively the dose-response relationships between lead exposure and HVA. Studies will be extended to plasma catecholamine measures, following development of appropriate high-performance liquid chromatographic electrochemical methods of detection (at Baltimore City Hospitals, in collaboration with Dr. David Thomas). A recent report, from Dr. Israel Hanin, University of Pittsburgh, indicates possible utility of measuring red blood cell choline concentrations in neuropsychiatric diseases involving dysfunction in central cholinergic pathways. Since our earlier work has shown that low level lead exposure is associated with decreases in high affinity choline uptake and release of acetylcholine from CNS nerve terminals as well as neuromuscular junction, we are developing a collaborative protocol with Dr. Hanin to study red cell choline metabolism in lead-exposed children.

#### B. Neurotoxic Aspects of Porphyrinopathies

Methods Employed: A new model of chemical and hereditary porphyrias was produced by administration of the recently isolated natural toxin succinylacetone. Succinylacetone was given to rats acutely by intraperitoneal administration and chronically for as long as 60 days by combined depot administration subcutaneously and implantation of continuously infusing minipumps. Parameters of heme synthesis were measured, using standard assay methods, in collaboration with the Metabolism Branch, NCI, and the Department of Pediatrics, Johns Hopkins Medical School (erythrocyte protoporphyrin, ALA dehydrase, ALA synthase, urinary and organ ALA). Parameters of GABAergic neurochemistry were measured (high affinity GABA uptake and synaptic GABA receptor binding) along with a sensitive indicator, developed in this laboratory, of neurobehavioral function related to the state of GABAergic neurotransmission, sensitivity to chemically-induced seizures monitored during continuous intravenous infusion of chemical convulsants.

Major Findings: One of the early toxic actions of lead at the biochemical level is the inhibition of heme synthesis. This cellular effect provides two markers which are widely used as biological indices of lead toxicity, elevated levels of red cell protoporphyrin, and decreased activity of red cell d-aminolevulinic acid dehydrase (ALAD). These effects are convenient to measure in erythropoietic tissue, but appear to occur in many heme-



synthesizing cells. The significance of altered heme synthesis in the toxicity of lead exposure is unknown. Our studies on the neurochemistry of lead poisoning in children demonstrated lack of correlation between urinary HVA and any indicator of altered heme synthesis. However, porphyriopathies, of which lead is an example, have a more general interest in neurology and toxicology. Several metals, aside from lead, as well as pesticides and other polyhalogenated hydrocarbons, inhibit heme biosynthesis. Frequently, this inhibition occurs at levels of exposure and body burdens below those associated with direct neurotoxicity. Chemical evidence of porphyria has been reported in clinical cases of exposure to kepone, polybrominated biphenyls vinyl chloride, and dioxin. In addition, there are several hereditary disorders of heme synthesis, notably the acute attack forms of porphyria, which are distinguished by neurotoxic manifestations. The causes of central and peripheral neurotoxicity in porphyria are at present unknown, but its striking similarity to lead poisoning has long been noted. Because of these similarities, it has been hypothesized that altered heme synthesis may in itself affect neuronal function. Because of the inability of lead to affect GABAergic neurotransmission directly (as reported last year), it may be that the GABA-toxic effects of lead exposure, which occur rapidly, even under conditions of acute exposure, are the result of heme-related actions of lead. As reported last year, we have shown that the heme precursor ALA inhibits the binding of GABA to synaptic receptors and the high affinity uptake of GABA by presynaptic nerve terminals. Both of these effects were demonstrated *in vitro*. It has been difficult to study the effects of ALA *in vivo*. Models of chemical porphyria have used acute, high doses of allylisopropylacetamide, sedative barbiturates, or toxic hydrocarbons such as hexachlorobenzene. These compounds, at the levels used, are very toxic in themselves and thus cannot be used to produce models of semichronic elevations in ALA. The barbiturates have actions on GABAergic neurotransmission which would confound interpretation. Administration of ALA itself is expensive and the pharmacokinetics of dosage are unknown, making it difficult to reproduce the conditions of sustained high levels of ALA in plasma. We have demonstrated that ALA can cross the blood-brain barrier, so that overproduction of this heme intermediate in peripheral organs (such as the erythron, liver, or kidney) can be expected to reach the CNS.

The Metabolism Branch, NCI, has recently provided us with a novel compound which inhibits heme synthesis at the same enzymatic step as lead. Succinyl acetone (4,6-dioxoheptanoic acid) was isolated by Dr. Donald Tschudy from the urine of patients with hereditary tyrosinemia, another disorder involving altered heme synthesis. Succinylacetone is of interest in oncology because of the role of porphyrins in tumor growth and metastasis." In collaborative studies, we have studied rats treated acutely and chronically with succinylacetone for effects on GABAergic neurochemistry and associated neurobehavioral changes. Prior work, with lead, identified high affinity GABA uptake by nerve terminals from cortex and basal ganglia as being a highly sensitive indicator of neurochemical toxicity, and lowered seizure threshold, to GABA antagonists, as an appropriate indicator of behavioral toxicity. Administration of succinylacetone for periods as short as one week produces significant inhibition of high affinity GABA uptake by cortex, caudate, and substantia nigra synaptosomes, as well as a significant decrease in the amount of picrotoxin required to elicit signs of CNS preconvulsive excitation. This is the first indication of heme-related neurotoxicity using an appropriate "pure" model of porphyria. (Succinylacetone by itself has no GABAergic

effects in vitro). Additional significance of these results is related to experimental cancer chemotherapy, since work by Tschudy and colleagues has shown that succinyl acetone, at least, in vitro, may inhibit growth of certain tumor cells. The potential neurotoxicity of succinylacetone must be assessed before any use in treating cancers clinically.

Projected course: Specific details in dose and exposure time will continue to be studied to determine more precisely the nature of succinylacetone-induced effects on CNS GABA neurotransmission in the rat. Structural analogs to succinylacetone are being synthesized by Dr. Tschudy; these will be studied as available to determine if heme effects are completely predictive of neurotoxic effects. The effects of reversing heme synthesis inhibition by administration of hematin will be studied, since this was successfully accomplished both clinically and experimentally in earlier studies with lead, in collaboration with Dr. J. M. Lamon, Scripps Clinic. Other types of chemical porphyrias will be studied to determine the interrelationships between heme synthesis inhibition and any GABA-related neurotoxicity.

## C. Neurotoxic Actions of Artificial Food Colors

Methods: In vitro and in vivo actions of the fluorescein type artificial food drug and cosmetic dyes have been studied. For in vitro studies, crude and highly purified preparations of CNS, PNS, and other tissues have been used in assays related to ion-translocating ATPases. For in vivo studies, several methods of exposure, ranging from chronic feeding to acute intracerebro-ventricular injection, have been used. Neurobehavioral outcomes measured include several spontaneously emitted "ethological" behaviors as well as highly quantitated and specific behaviors.

Major Findings: Erythrosin B, or tetraiodofluorescein, commonly known as U.S. Food, Drug, and Cosmetic dye Red No. 3, is a widely used artificial coloring agent in foods; as such, it is currently under widespread study as part of the concern focussed on artificial additives as causative agents in children's behavior and learning disorders.

### 1. In Vitro Studies:

We reported earlier that in vitro erythrosin B can block the sodium-dependent uptake of dopamine into synaptosomal; this inhibitory action is not limited to dopamine uptake but common to many sodium-dependent processes and have been confirmed by several other laboratories. We have also demonstrated that erythrosin and three of its congeners (rose bengal, also a FD and C dye, eosin Y, and di-iodofluorescein) are highly potent inhibitors of a Na,K-ATPase unique to brain tissue. These compounds do not affect red cell, kidney, or eel electroplax ATPase. In the brain, the ability of erythrosin to inhibit so-called high affinity glycoside binding is parallel to the incidence of this site in specific regions and neural structures. Thus, the cortex, hypothalamus, hippocampus, and fornix contain relatively high amounts of this binding site, and these are the areas where erythrosin is most effective at inhibiting ATPase activity; the callosum and basal ganglia contain relatively less sites, and erythrosin has less effect on ATPase activity. The optic nerve is sensitive to erythrosin; the trigeminal and phrenic nerves and myenteric plexus are insensitive and a similar parallelism prevails. In the CNS, a purified myelin fraction (prepared by Dr. Richard Quarles) contains

only the low affinity binding site and its ATPase activity is insensitive to erythrosin. These results thus suggest that erythrosin B is likely to be a powerful probe for studying one form of Na,K-ATPase which maybe selectively localized on certain structures of the central nervous system.

Erythrosin B also appears to effect another ion-translocating ATPase, the Ca-Mg ATPase activity of rabbit muscle sarcoplasmic reticulum. In a recent study of the photooxidative degradation of sarcoplasmic reticulum (SR) proteins by erythrosin B and rose bengal, it was found that erythrosin B significantly inhibited calcium transport at  $\mu\text{M}$  concentrations in the absence of light. In collaboration with Dr. Duncan Haynes, Univ. of Miami Medical School, we have studied in detail similarities between inhibition by erythrosin of the SR and brain ion-translocating ATPases. Calcium transport, ATPase activity and binding of erythrosin B and ouabain to purified SR vesicles were studied by published methods. We have found that erythrosin B inhibits  $\text{Ca}^{2+}$ -transport with an  $\text{IC}_{50}$  of  $\sim 1 \mu\text{M}$ , inhibits  $\text{Ca}^{2+}$ -activated ATPase activity with the same  $\text{IC}_{50}$  and binds to the tissue by a saturable mechanism with a  $K_d$  of  $\sim 40 \text{ nM}$ . Ouabain and oligomycin do not inhibit this enzyme; thus the effects of the dye are not a reflection of contamination by Na-K or mitochondrial Ca-ATPase. The dye-promoted effects are independent of the presence of light. Dye binding and ATPase activity are also inhibited by the halogenated fluorescein analogs Rose Bengal, Eosin Yellow, and Eosin Blue in the descending order of potency. The parent compound fluorescein is without effect.

We have obtained custom-synthesized [ $^{14}\text{C}$ ]-erythrosin B from Dynapol and with this ligand we have explored further the nature of interactions between the dye and neural membranes. Erythrosin B appears to bind to crude and purified brain synaptic membranes in a saturable, ion-dependent, "receptor-like" manner. Its  $K_d$  is in the range of 5-50 nM, and the number of binding sites (in crude preparations of synaptic membranes) is approximately 100 fmol/mg protein. With purification of membrane preparations (by standard sucrose gradient methods), the affinity is decreased somewhat, and the number of binding sites greatly increased. The distribution of erythrosin-binding sites follows that of high affinity [ $^3\text{H}$ ]-ouabain binding sites, with greatest enrichment in synaptosomal membranes and no significant presence in purified synaptosomal mitochondria. The use of this ligand has further clarified the nature of interactions between erythrosin and the glycosides. Although erythrosin is the most potent inhibitor of glycoside binding yet identified outside of the glycosides themselves, no glycoside inhibits the binding of erythrosin. This apparent lack of complementarity, as well as important differences in temperature-, energy-, and ion-dependence, serves to differentiate the sites of action of these compounds. The extensive structure-activity work done by many groups on the glycosides would preclude the possibility that fluorescein-like compounds could "fit" molecularly into the glycoside receptor. Other components which have been identified in the ATPase complex are ion-binding sites for Na, K, and Mg; an ATP-binding site; and a site for transducing the inhibitory effects of vanadate. Erythrosin does not bind to the ion sites, nor to the ATP binding site. There may be some interactions between erythrosin and vanadate, but the relatively low level of potency of vanadate as a displacer of erythrosin argues against any direct, competitive interaction or the same site of binding. Other agents which affect ATPase activity, such as chlorpromazine, ethanol, ethacrynic acid, dithiothreitol, EDTA, oligomycin, and lead, do not affect the binding or inhibitory actions of erythrosin. Other parts of this project on the in vitro



biochemistry of erythrosin are reported in the project Z01 02451-01, In Vitro Approaches to Neurotoxicity.

2. In vivo studies of the effects of ingestion of Erythrosin B on animal behavior and neurochemistry.

In order to study the in vivo effects of artificial food dyes on animal behavior and neurochemistry, animals were given 2mg/kg Erythrosin B per day in their drinking water. This dose is based on the estimated average intake for children in the United States. Two behavioral tests were selected for measurement: 1) open field behavior (a test of general activity level, exploration, and reactivity or emotionality); and 2) nest building (an easily elicited activity of adaptive significance). To measure open field behavior animals were placed in the corner of a square plexiglas open field (75 cm. per side with the floor divided into 16 equal sections by grid marks). For three minutes the number of lines crossed, rearings, grooming activity, and fecal boli were recorded. Nest building behavior was tested by placing cotton batting in metal dispensers into cages of individually housed animals. The amount of cotton pulled (determined by weight) and the quality of nest built (determined by visual observation) was recorded.

After in vivo administration, neurochemical measures (high affinity binding of a cardiac glycoside, ouabain, and Na,K-ATPase activity) were made in crude preparations of cortex synaptic membranes. Binding of [ $^3$ H]-ouabain to the high affinity sites of crude synaptic membranes from cortical tissue was measured by incubation of the tissue preparation with low concentrations of ligand (0.5 to 4.0 nM). Na,K-ATPase activity was measured in these same tissue homogenates by spectrophotometric quantitation of the production of Pi from ATP.

Sprague-Dawley rats administered erythrosin B (2mg/kg/day in their drinking water from 8 weeks of age to 20 weeks of age) were not different from controls in fluid consumption or body weight. After 3 months of chronic erythrosin B exposure, open field activity was recorded for three minutes during the middle of the animals active cycle (midnight). No differences were found between the experimental and control groups in rearing, defecation, or activity. The rats ingesting red No. 3 spent more time grooming during the open field test period than the control rats. During the last four weeks of erythrosin B administration, nest building was measured. The control animals used 85 percent more cotton than the experimental animals and the nests built by the animals exposed to erythrosin B were not as well constructed as those built by the control animals. Animals were decapitated at 20 weeks of age, brains removed, and high affinity ouabain binding and Na,K ATPase activity were measured in cortical tissue preparations. No differences were found between the experimental and control rats in high affinity binding of ouabain or Na,K-ATPase activity in crude cortical tissue preparations.

3. Behavioral effects induced by intracerebroventricular injections of erythrosin B and related compounds

The potent in vitro actions of erythrosin B on release and reuptake of several neurotransmitters and on brain enzymes contrast sharply with its weak behavioral effects after systemic administration to rats. From such behavioral evidence, it has been suggested that erythrosin B does not cross the blood-brain barrier. However, this barrier might be crossed and under appropriate conditions, and then erythrosin B might be shown to possess neuroactive and/or neurotoxic properties. To answer this question, we

assessed the behavioral effects of erythrosin B and related xanthene compounds injected by a more direct route, i.e., into the lateral cerebral ventricles of chronically implanted, freely moving rats.

In vitro experiments have shown that: (a) erythrosin B blocks one type of neuronal Na-K ATPase, i.e., one which constitutes the high-affinity binding site of the digitalis-like compound ouabain; (b) in this regard, erythrosin B and ouabain are equipotent; and (c) erythrosin B and related fluorescein-derived xanthene compounds can be classified into those that act on neuronal Na-K ATPase (e.g., erythrosin B) and others that are devoid of such action (e.g., fluorescein, phloxin, eosin B). If the in vitro ouabain-like action of erythrosin B on brain tissue has behavioral relevance, then intracerebral injection of erythrosin B or ouabain should produce similar behavioral effects at equimolar concentrations. In fact, erythrosin B could be expected to be more potent because of its high lipophilicity in contrast to ouabain, which is one of the least lipophilic digitalis-like glycosides. If in vitro ouabain-like actions of xanthene compounds are responsible for their behavioral and/or neurotoxic effects in vivo, then their relative affinities for cerebral Na-K ATPase in vitro should be reflected in their relative behavioral pharmacologic and neurotoxic potencies.

Our experiments on intracerebroventricular (ICV) injection of erythrosin B provide the first and most direct evidence aimed at testing the validity of claims regarding its neuroactive and neurotoxic properties.

Stereotaxic procedures were used to implant adult male rats with 26 Ga stainless steel cannulae in the lateral cerebral ventricles. Three to 7 days later, rats were injected by means of a Hamilton microliter syringe attached to PE 20 tubing and a 30 Ga stainless steel injection cannula with one of several xanthene compounds dissolved in 10 microliter of saline, or with the vehicle alone. When multiple injections of a substance were given, successive injections were separated by 30 minutes. Immediately after an ICV injection, rats were placed in an 18" X 18" X 24" Plexiglas open field and their behaviors recorded on a videotape system for later quantitative analysis. States of akinesia were further characterized by testing for signs of neuroleptic-like and opiate-like catalepsy developed by De Ryck. Locomotor activity was further analyzed in terms of its topography, directionality and sensory controls. Finally, the duration and intensity of distinct behavioral stages were determined following ICV injection of a wide range of dosages of erythrosin B and related compounds. Rats were maintained and behaviorally tested for up to 2 weeks after the injection(s). Under deep barbiturate anesthesia, brains were fixated by cardiac perfusion with saline and 10 percent formalin and prepared for frozen tissue sectioning and staining in order to histologically verify the cannula placements. Other histology experiments were designed to check the distribution of the dye throughout the ventricular system of the brain, the time span of their visible presence in the brain, and their tendency to be washed out of the brain by saline perfusions.

Successive ICV injections of 0.5, 4 and 20 nanomoles of fluorescein or erythrosin B did not produce behavioral changes in the open field, except for irregular breathing and teeth gnashing after 20 nanomoles (24.5 nanomoles cumulatively) of erythrosin B. However, within 1-3 minutes following 200 nanomoles (224.5 nanomoles cumulatively) of erythrosin B, about 25 percent of the rats showed short-lasting episodes (5-15 minutes) of compulsive running. This finding was replicated in another group of rats which received 200 nanomoles of erythrosin B as their first injection. Compulsive running was

typically preceded by symptoms of neuroleptic-like and opiate-like catalepsy; and followed by a form of passive or depressed akinesia consisting of a diminished muscle tone (i.e., general muscular weakness and reduction in antigravity tone), a coarse lateral body sway (typical of rats going into or recovering from barbiturate or chloral hydrate anesthesia), sensory neglect and limb-placing deficits. Erythrosin B-treated rats which failed to develop running bouts only showed this form of akinesia devoid of cataleptic phenomena. After a single injection of 400 nanomoles of erythrosin B, all rats invariably produced the following behavioral sequence for 30-60 minutes after injection: Akinesia - Neuroleptic-like and Opiate-like Catalepsy - Compulsive Running - Akinesia. Thus, the ED50 for compulsive running and related behavioral states required between 200 and 400 nanomoles of erythrosin B. (At these dosages, fluorescein did not produce any behavioral change).

In contrast, ICV injection of only 4 nanomoles of ouabain sufficed to produce compulsive running lasting for 3 hours. This state was accompanied by spontaneous or stimulus-elicited tonic-clonic seizures, yielding the behavioral sequence: Akinesia - Neuroleptic-like and Opiate-like Catalepsy-Compulsive Running - Tonic-clonic Seizures - Interictal Depression - Compulsive Running. These results also suggested that compulsive running constitutes one of the behavioral precursors to convulsions. Therefore, contrary to a low dose of ouabain, high dosages of ICV erythrosin B only produced preconvulsive behavioral states, without producing seizures.

In order to determine the dose-dependence of erythrosin B-induced compulsive running, rats were treated with successive injections of 200 or 400 nanomoles of erythrosin B. Only the first injection produced running episodes. Additional injections were ineffective and resulted in akinesia accompanied by severe hyperthermia (rectal temperatures of 40-42°C rectal temperature). These experiments also demonstrated the remarkably low lethality caused by very high dosages of ICV erythrosin B. For instance, only 1 out of 6 rats treated cumulatively with 1.2-1.6 micromoles of erythrosin B died 2 days later (probably due to prolonged hyperthermia).

At dosages of ouabain which produced seizures, rats recovered to normal within 24 hours. However, at dosages of at least 2 orders of magnitude larger, erythrosin B, which did not produce tonic-clonic seizures, produced long-lasting behavioral effects, i.e., tactile hyperreactivity lasting up to 1-2 weeks after treatment.

Rats treated with 400 nanomoles of eosin B, which, like fluorescein, lacks *in vitro* ouabain-like actions, presented with status epilepticus within 15 minutes after injection and died within 30-45 minutes.

These experimental findings warrant the following conclusions:

- (a) When the food dye erythrosin B is directly injected in the lateral ventricles of freely moving rats, only very high dosages yield prominent behavioral effects. This result indicates that, contrary to its *in vitro* actions, erythrosin B is a weak neuroactive agent in vivo. Furthermore, at dosages 100 times larger than those of ouabain, erythrosin B only produces a fraction of ouabain-induced behaviors, i.e., states preceding tonic-clonic seizures. The structurally related xanthenes, fluorescein and eosin B, lack ouabain-like activity *in vitro*. Whereas the former is without behavioral effects, the latter produces severe behavioral toxicity with immediate 100 percent lethality at equimolar dosages of erythrosin B, which only lead to a low-level (17 percent) delayed



lethality. This triple dissociation suggests that the behavioral effects so far observed of erythrosin B cannot be attributed to a specific ouabain-like action on one type of cerebral Na-K ATPase.

- (b) Several behavioral observations indicate that nonspecific neurotoxic factors may account for the behavioral effects of erythrosin B. In those rats which showed erythrosin B-induced compulsive running, no obvious dose-dependent differences in its amount or duration could be discerned between treatments with 200 versus 400 nanomoles of erythrosin B. Of successive injections, only the first one caused running. The other injections did not produce this short-lasting excitatory effect. They also failed to enhance the (low) rate of lethality (e.g., 4 injections of 400 nanomoles of erythrosin B did not result in a greater lethality rate than a single injection).

The behavioral states induced by these high doses of erythrosin B resemble those produced by central injection or electrolytic deposition of metallic ions. Because very high dosages of erythrosin B are required to yield these states, it is conceivable that these behavioral effects represent an acute neurotoxic action of a nonspecific, irritative (excitatory) type. The chronic effect of hyperreactivity resembles the long-term effects induced by electrolytic destruction of the septum or anterior hypothalamic/preoptic area, i.e., structures adjacent to the lateral ventricles and the third ventricle, respectively. It is possible that behaviorally effective dosages of erythrosin B cause irreversible cellular destruction. The 1-2 week recovery from tactile hyperreactivity may be attributed to unaffected cells in periventricular areas, which, as other evidence suggests, are only partially affected by erythrosin B. For instance, when cannulae were located in the lateral ventricles, 200 nanomoles of erythrosin B did not produce tonic-clonic seizures. When their implantation site was moved 500  $\mu$  more medially, into the fornix, the same dose readily produced seizures. (It should be noted that ouabain yielded seizures after intraventricular injection at doses 50-100 times less than erythrosin B). Thus, contrary to ouabain, erythrosin B does not easily reach structures distant from the ventricular bed. Cells so affected might show transient or irreversible metabolic disturbances resulting in an initial irritative (excitatory) effect followed by a reversible or irreversible depression of activity. The seemingly "protective" effect of the first injection of a high dose of erythrosin B against subsequent ones argues in favor of such a view.

- (c) The *in vitro* neuroactive effects of erythrosin B are most likely not related or restricted to its nonspecific neurotoxic properties. In vitro experiments, using synaptosomal fractionation procedures, may provide accessibility of erythrosin B to cellular compartments which it never reaches in *in vivo* conditions. Our experimental results indicate that it would be unrealistic to expect major neurotoxic effects from normal ingestion of erythrosin B through colored foods and drinks. First, the dye may bind to peripheral structures or be eliminated before ever reaching the brain. And secondly, it would be unreasonable to assume that food- and drink-related ingestion of

erythrosin B would ever reach the human brain at concentrations required to produce the behavioral toxic effects experimentally demonstrated in our rats. If it were unequivocally substantiated that a subset of children are hypersensitive to food dyes, our experiments make it unlikely that central effects of erythrosin B are responsible.

Projected course: The nature of erythrosin interactions with synaptic ATPase will be further studied, both in terms of neurotoxic potential of the dye and in terms of its basic utility as a probe for brain neurochemistry. In collaboration with Dr. R. W. Albers, Laboratory of Neurochemistry, NINCDS, studies are being developed to copurify glycoside and dye-binding sites and to characterize these entities molecularly. In addition, comprehensive regional studies of dye binding and enzyme inhibition will be completed.

Experimental evidence is needed to adequately demonstrate that erythrosin B-induced behavioral states, in particular compulsive running bouts, are preconvulsive. Rats will be chronically implanted with neocortical and hippocampal electrodes in addition to ventricular cannulae, and the EEG correlates of erythrosin B-induced behaviors will be monitored. erythrosin B-treated rats will also be pretreated with anticonvulsant agents (e.g., diphenylhydantoin) to test the preconvulsive effects of erythrosin B. The behavioral neurotoxicology of erythrosin B will be compared with the neurotoxic potencies of other fluorescein-derived xanthene compounds, such as Rose Bengal, Eosin Y, Mercurochrome, and Rhodamine B. Such a structure-activity approach will allow a more precise definition of the relative neurotoxicity of erythrosin B in relation to other xanthene dyes. Natural xanthene compounds are rare, but structurally related anthraquinone substances, several of which are coloring matters, have been isolated from fungi, lichens and plants. Their behavioral pharmacologic and neurotoxic properties are unknown. We would like to determine whether these natural principles have any specific neurobehavioral effects. Also, a by-product of these studies has been the definition of some of the low-level neurotoxic signs of ouabain exposure; these will be further characterized to define and understand the reported side-effects of digitalis administration.

Preliminary studies suggest that erythrosin may be able to penetrate the placental barrier; the finding of inhibitory effects on muscle sarcoplasmic reticulum ATPase indicates that penetration of the blood-brain barrier may not be necessary for some adverse effects to occur.

#### D. Investigations of variable sensitivity to neurotoxins

##### Project Description:

Objectives: (1) to define populations or individuals that might be at increased risk to the effects of neurotoxins, (2) to use specific variability in central nervous system function, anatomy, and/or neurochemistry to elucidate mechanisms of action of neurotoxins.

Methods and approaches employed: Individual variation in nervous system may predispose some individuals to increased risk of damage, impairment, or degeneration after exposure to neurotoxins. We are presently studying variation in the central nervous system due to age, heredity, or previous experience in relationship to some of the current interests of the NTS (i.e.,

neurotoxic effects of heavy metals and erythrosin B). The sources of variation presently being explored are: (1) fetal and neonatal development, adult maturation, and aging; (2) screening of apparently normal genetically inbred rodent populations for neurochemical variation, and investigations of genetically defined rodent lines known to differ in central nervous system function, anatomy, and/or neurochemistry; (3) prenatal exposure to low levels of alcohol as a possible environmentally induced factor which potentiates insult or injury by other neurotoxins (e.g., lead or erythrosin B) later in life.

### Major findings:

#### 1. Age differences:

We have previously presented data on the binding of the cardiac glycoside, ouabain, to two (ligand concentration specific) sites in synaptic membrane preparations from rat brain but to only one site in other rat tissue preparations. We have demonstrated inhibition of Na,K-ATPase activity, dopamine uptake, and high affinity (but not low affinity) ouabain binding by erythrosin B in crude synaptic membrane preparations from rat cortex. There are clinical, anecdotal and experimental claims that food dyes have neurobehavioral effects in children and young animals. In order to evaluate the possibility that age variation in Na,K-ATPase structure and/or activity may confer susceptibility to erythrosin B toxicity, we are investigating age and Na,K-ATPase relationships by measuring Na,K-ATPase activity and ouabain binding to crude synaptic membrane preparations obtained from rats of various ages. Na,K-ATPase in cerebral cortex from male genetically heterogeneous (NIH/N) rats is being studied. Two age ranges are being examined: the neonatal development stages (10, 15, 20, and 30 days old) and adult maturation (30, 60, 90, 120, and 240 days old). Analysis of preliminary data by saturation curves and Scatchard analyses of ouabain (0.25 - 300 nM) binding to crude synaptic tissue preparations from cerebral cortex indicate two ouabain binding sites ( $KD_1 \approx 1.3$ ,  $KD_2 \approx 80.0$ ) for all age groups except rats 10 days of age where only one binding site ( $KD \approx 35.0$ ) is indicated. The development of myelination in the cortex at 14 days of age as well as other data from this laboratory on the presence of only low affinity ouabain binding in myelin from tissue cultures suggest that this age difference may be related to myelination in the cortex and to a morphological compartmentation of differential ouabain binding in the central nervous system.

#### 2. Genetic variations:

Eighty inbred rats (four from each of twenty different strains) were exposed to chronic administration of erythrosin B (2mg/kg/day) in drinking water from 6 to 12 weeks of age. Open field activity, and high affinity ouabain binding and Na,K-ATPase activity in crude synaptic preparations were measured as described above, in order, to screen for genetic differences in response to erythrosin B. No differences between experimental and control rats were found for body weight, fluid consumption, high affinity ouabain binding, or Na,K-ATPase activity. Differences in open field activity were found between experimental and control animals only in the Brown Norway strain. Acute 50 mg/kg administration of erythrosin B by gastric intubation to 20 day old Brown Norway rats did not result in differences in open field



activity, high affinity ouabain binding or Na,K-ATPase activity in crude synaptic preparations from brain cortex.

If there is a threshold of ATPase activity below which nervous system function is impaired, individuals at the low end of normally occurring variability in enzyme activity may be affected by inhibition of enzyme activity by neurotoxins. We screened twelve inbred rat strains for variation in Na,K-ATPase activity in cortical synaptic membrane preparations. A two-fold difference in brain Na,K-ATPase activity (measured spectrophotometrically by determination of Pi from ATP) was found between the highest (MNR) and the lowest strain mean activity levels (WN). The inheritance of cortical Na,K-ATPase activity is being studied in these two strains of rats. Significant differences in high affinity ouabain binding were not demonstrated among the twelve inbred rat strains screened. However, substantial daily variation in data from ouabain binding assays may obscure small differences in ouabain binding to Na,K-ATPase among groups of animals.

We previously screened 20 different inbred rat strains for differences in neurochemistry. Measurement of liver and brain monoamine oxidase (MAO) activity in these inbred strains provided data on the relationship between MAO-A and MAO-B within diverse individuals. Correlations between tyramine and serotonin deamination (0.78), between tyramine and phenylethylamine deamination (0.83), and between serotonin and phenylethylamine deamination (0.33) reinforce evidence for two different forms of monoamine oxidase which can be distinguished by substrate preference. No strains were found in which there was an inverse relationship between MAO-A and MAO-B activity. A high correspondence between activity levels from liver and brain homogenates was also found. Whether this is a reflection of the inheritance of similar enzymatic forms of MAO or a suggestion that the same factors modulate the activity of both the A and B forms of monoamine oxidase can not be ascertained from the present data.

### 3. Environmental predisposing factors:

Previous exposure to low levels of neurotoxins may cause minor alterations in the central nervous system that do not have observable effects on normal function or behavior but may predispose the individual to increased susceptibility to neurotoxic insult by small amounts of other neurotoxins later in life. We are now investigating the possibility that animals prenatally exposed to low levels of alcohol in utero may be at increased risk to erythrosin B neurotoxicity when exposed to that toxin post-natally. Rats exposed to low levels of alcohol prenatally will be assessed for abnormal behavior in the prenatal period and young adulthood with a series of ethological and psychological tests.

Previous data from this laboratory have indicated that the *in vitro* response of Na,K-ATPase to ethanol is concentration dependent, the graphic presentation of the effects of ethanol on ATPase activity is a U-shaped curve. The mechanism of action of ethanol on Na,K-ATPase activity may be related to the action of alcohols in changing the fluidity of biological membranes. This action is being investigated in relationship to its effects on an interaction and inhibition of Na,K-ATPase by ouabain and erythrosin B. In order to provide diverse systems in which to study this relationship, this investigation is comparing normal animals with those pretreated with alcohol prenatally and/or postnatally, and rodent lines genetically developed by selective breeding for differences in alcohol-related behaviors.

Proposed course:

Present evidence suggests that although a vast majority of the United States population is exposed to food dyes, only a small number of individuals display neurobehavioral effects. Data from animal experimentation are, likewise, variant in their demonstration of behavioral alteration by food dyes. It seems likely that certain individuals or groups of individuals may be susceptible to neurobehavioral changes in response to food dyes by predisposing factors related to their age, heredity, and/or environmental milieu.

Several strains with high, low, and intermediate MAO activity (as determined from the preliminary screening project) are being used in a detailed analysis of the mechanisms of the inheritance of MAO activity, and to study the effects of manganese on MAO activity.

We are continuing to investigate the relationship between age and specificity for differential ouabain-binding sites. This project will be expanded to include the study of Na,K-ATPase activity levels, ouabain and erythrosin binding and inhibition in different neuronal cell lines from tissue culture, regional distribution in rat brain, isolated cell types from brain tissue, and in mutant rodent lines characterized by myelin degeneration.

Clinical data have been presented on reduced Na-K pump activity in the red blood cells of patients with idiopathic obesity. This reduced Na,K-ATPase activity is not secondary to obesity per se. "Acquired" forms of obesity (e.g. due to pituitary insufficiency resulting from intracranial lesions) show the same spectrum of Na-K pump levels seen in normal weight controls. That red cell findings may reflect similar changes in additional cell types is suggested by a high correlation in pump status between red cells and mononuclear white cells. Alteration of Na,K-ATPase activity in idiopathic obesity is being investigated in mutant rodent lines (C57BL/6J-ob/ob and LA/N-cp/cp) as a possible model to elucidate the dynamics of Na,K-ATPase interactions with ouabain and erythrosin B.

#### E. Drug-induced Immobility States as Animal Analogs of Parkinson's Disease

**Methods:** In neuroleptic catalepsy, tonic postural reactions subserving static support are enhanced at the expense of locomotor reactions. For instance, when haloperidol-treated rats are pushed forward, backward or sideways, they show exaggerated rigid bracing reactions which resist the force of displacement and prevent transfer of body weight in the direction of displacement. Such bracing reactions may also appear spontaneously, and are associated with attempts at forward locomotion or with its sudden arrest. They can be considered an animal analog of Parkinsonian freezing reactions. Electromyographic recordings have added to behavioral evidence that haloperidol-induced bracing reactions are static supporting reactions: cataleptic bracing consists of simultaneous contractions of antagonist flexor and extensor muscles in the limbs, a pattern underlying the Positive Supporting Reaction of Schoen and Magnus. Furthermore, neuroleptic-induced bracing reactions appear to be under exaggerated vestibular control, which we have interpreted as supraspinal compensation for the well-documented impaired spinal inputs from muscle afferents and for a depressed gamma loop after neostriatal dopaminergic blockade or depletion. In order to determine whether or to what extent neuroleptic-induced bracing/freezing reactions are mediated by vestibular reflexes, we deemed it of interest to test the effects of unilateral and bilateral vestibular deafferentation on these reactions.

It is no easy task to surgically access the bulla and remove the labyrinth without unintended damage to facial muscles, tendons, blood vessels or nerves other than the eighth cranial nerve. For chronic behavioral purposes, we considered it indispensable that the vestibular damage be properly localized and confined; and that the technique to produce it be highly reliable. We resolved this problem by developing a stereotaxic technique for vestibular deafferentation. While histologic proof of its validity is being gathered, the behavioral effects of unilateral damage clearly indicate that our new technique is both valid and reliable. Behavioral analyses have involved detailed analyses of videotaping motor performance as described below.

Major Findings etc: The seemingly bizarre rolling movements produced by unilateral vestibular deafferentation are released asymmetric righting movements towards the lesioned side. Thus, in the air, such animals are unable to attain a symmetrical preparatory landing position, but instead continue to right themselves. Likewise, in water, under conditions of reduced support and provided that they do not contact walls, these animals perform continuous, self-perpetuated righting cycles. When they are suspended by the tail, a complex conical spinning movement ensues that can be shown to result from the superimposition of all three movement components involved in normal righting, but whose asymmetric combination imparts a conical spin to the body and tail. Slow motion and frame by frame analysis of videotapes filmed at 50 fps have enabled us to isolate the different movement components making up these forced movements and to conclude from analyses of their latencies that they are components of self-sustained air righting reactions.

As in normal contact righting, uncontrolled rolling on the ground is considerably slowed down. Thus, tactile and/or proprioceptive stimulation arising from body contact and/or support has an inhibitory effect on righting. When unilaterally damaged rats showed repeated rolling movements on a smooth Plexiglas surface, such movements were completely inhibited by transferring them to a grid floor to which they intensely clung. Thus, tonic grasp inhibits righting.

We tested these conclusions more directly, and made two new discoveries. When continuous pressure was applied to a small skin area (i.e., a miniature alligator clip in the nape, producing no physical restraint of movement), excessive righting movements in rats with unilateral vestibular damage were abolished. Righting movements in response to tail suspension were also inhibited by allowing unilaterally damaged rats to grasp a small object in both forepaws (e.g., a wooden applicator). Thus, skin pressure, tonic grasp and support exert inhibitory influences over movement components involved in righting. Or, stated otherwise, they all block the uncontrollable forced movements produced by unilateral vestibular damage.

Projected Course: Having first verified the validity and reliability of our new technique of vestibular deafferentation, we will now prepare rats with bilateral vestibular deafferentation to evaluate the effects of such damage to the neuroleptic bracing/freezing reaction. We would like to pursue this analysis by testing the effects of spinal deafferentation on neuroleptic-induced exaggerated supporting reactions, as well as on morphine-induced rigidity in otherwise unrestrained, freely moving rats. These projects will also involve the use of sensory neurotoxins (e.g., adriamycin; capsaicin) and chronic electromyographic recording from limb musculature of freely moving rats.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 NS 02451-01 ODIR																																																				
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COOPERATING UNITS (if any) University of Miami Medical School, Miami, FL 33101; Duke University Medical School, Durham, NC 27706; Max Planck Institute for Biophysical Chemistry, D-3400 Goettingen, F.R. Germany; SNB, NINCDS; CNB, NIMH																																																						
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<p>Several <u>in vitro</u> systems were explored for their applicability to the testing of (suspected) neurotoxic substances, such as Erythrosin B (FD and C Red 3) (EB) an artificial halogenated <u>fluorescein derivative</u>. The dye is also useful for solubilizing and partially purifying ATPase from rat brain cortex. It also inhibits ATPase and calcium transport activity of rabbit muscle sarcoplasmic reticulum. <u>In vitro</u> toxic effects are also monitored by studying the growth of chick dorsal root explants.</p> <p>Storage and release of catecholamines from adrenal medullary cells are affected by a variety of heavy metals, partially through interference with calcium-specific mechanisms involved in release of the neurotransmitter. The calcium-promoted fusion of isolated chromaffin granules, and its inhibition by various heavy metals, is being studied as a model process for exocytotic release of catecholamines <u>in vivo</u>. The kinetics of calcium-promoted fusion have been studied using <u>fluorescence energy transfer techniques</u>.</p>																																																						

### Project Description

Objectives: Although a large number of compounds have been identified as neurotoxic agents, there is relatively little understanding of the basic biochemical mechanisms which underlie specific actions of toxins on components of neurons and their target cells. Presently, identification of toxins and assessment of toxic potential are often labor-intensive and costly processes involving whole animal experiments which must run over long periods of time. Most of the diagnostic tests for impaired neurological function involve behavioral testing since little is known of the underlying biochemical dysfunction. This project has two long term goals: (1) to establish reliable paradigms for measuring and predicting damage to the nervous system by in vitro tests, which could be used to screen for suspected neurotoxins or augment those already in use, and (2) to understand the action of specific identified neurotoxins at the cellular and hopefully the molecular level. For these reasons the effects of various known and putative neurotoxic substances on several well characterized cellular and subcellular systems are being investigated.

### Projects:

#### I. Erythrosin B (FD and C Red 3) and related dyes.

##### A. Solubilization and purification of dye-binding sites from rat brain

Purpose: It has been established by this laboratory that the food and cosmetic dye, Erythrosin B inhibits uptake of neurotransmitters into isolated synaptosomes, inhibits the Na-K ATPase of these particles and selectively binds to a highly specific site on the synaptosomal membrane (see "Animal Models of Neurologic Disease", Z01 02264-05). There is growing evidence from this laboratory and others that the brain contains two Na-K ATPase isozymes which differ slightly in molecular weight and significantly in their affinity for ouabain. The dye-binding site seems to be located on the enzyme characterized by a high affinity for glycoside-binding and greater sensitivity to glycoside inhibition of ATP catalysis. Therefore, the dye may serve as a highly specific probe to study the function of this enzyme. These studies utilize [ $^{14}$ C]- radiolabelled erythrosin B to follow the detergent solubilization and subsequent purification of the dye receptor. Once isolated, the material can be characterized and compared to what is known about other aspects of Na-K ATPase activity. The properties of the dye-binding site can be characterized and compared to what is known about other activities and receptor sites on the Na-K ATPase complex.

Methods: The ability of various detergents to solubilize dye binding and ATPase activities from membrane fractions of rat brain cortex are assessed by respectively precipitating soluble dye/receptor complexes using the gamma globulin-polyethylene glycol procedure and well-established enzyme and ligand-binding assay procedures.

Major Findings: Several non-ionic detergents can solubilize the activities, although they are destroyed by strong ionic detergents. Ouabain-binding activity is very sensitive to detergent treatment but dye-binding and ouabain-sensitive ATPase activity often survive. This result complements the results of Silbergeld on unpurified brain tissue, which suggest that the



binding sites for these two ligands are separable. Solubilized ATPase activity is inhibited by the dye and several iodinated and brominated analogs but not by the parent compound, fluorescein. Unlike the native membrane-bound enzyme, there is no requirement for the presence of Mg-ATP for dye-binding to the solubilized material. Chromatography on Sephadex G-75 increases the specific activity without changing these properties and has established that the solubilized complex has a molecular weight of greater than 75,000 daltons.

Projected Course: The solubilized material will be further purified by affinity chromatography to answer the question of whether the dye-binding and ATPase activities are separable. The purified material will be used as an antigen for the production of monoclonal antibodies, which in turn will be used to identify and characterize dye receptors in situ. In collaboration with Dr. Wayne Albers, the similarities between the dye and ouabain receptor sites will be investigated.

#### B. Inhibition of NGF-stimulated neurite outgrowth by Erythrosin B

The nerve growth factor (NGF)-stimulated outgrowth of neurites from explanted 8-day chick dorsal root ganglia has been extensively characterized as a model for differentiation of the nervous system in vitro. A new, improved culture system for growing the ganglia was developed and the possible effects of halogenated fluorescein derivatives were tested.

Procedures: A new set of culture conditions was employed, in which the excised ganglia were polymerized into a matrix of native collagen rather than clotted chicken serum. Dyes and other factors were added to the medium before polymerization and their effect on neurite outgrowth and other parameters of cell growth and migration compared to controls.

Major Findings: The new culture conditions produced excellent outgrowth of neurites. In some cases cultures were observed for up to four days and neurites of length greater than 2 mm were seen. It was established that erythrosin B and other iodinated and brominated analogs significantly inhibit the NGF-stimulated differentiation of the chick sensory ganglion explants with an  $IC_{50}$  of  $5 \times 10^{-5}$  M. This effect can be differentiated from an inhibition exerted by ouabain, and is mostly due to photo-oxidation of the NGF. Thus the dye has neurotoxic effects other than enzyme inhibition. However cultures set and incubated in the dark also showed some retardation in growth of neurites, which suggests that toxic effects are not confined to single mechanisms.

Projected Course: The photo-oxidation of NGF by the dye was well documented and will not be pursued. However if culture facilities can be found, the use of this and other nervous system derived cell and tissue cultures will be tested for their possible use as screening of suspected neurotoxic agents (see below).

C. Comparative studies of the binding of erythrosin B and its derivatives to Na-K ATPase using time-resolved phosphorescent anisotropy measurements (S.J. Morris and T.M. Jovin, Chairman, Department of Molecular Biology, Max Planck Institute for Biophysical Chemistry, Goettingen, F.R. Germany).

**Purpose:** As noted above, erythrosin B inhibits the activity of brain Na-K ATPase. The phosphorescent properties of the halogenated dyes have been exploited by several groups by noting the changes in the rotational half-life and anisotropy of the dye when it is covalently bound to various membrane proteins including kidney ATPase. The object of the present study was to determine if the binding of erythrosin B to the Na-K ATPase of Torpedo electric organ and guinea pig brain could be detected by this methodology. If so, then the changes detected should give us information as to the nature of the binding site and the interaction of the dye with membranes.

**Methods:** Phosphorescence anisotropy and rotational half-times of free and non-covalently bound erythrosin B were measured using the pulsed laser phosphorescence spectrophotometer constructed by Dr. T.M. Jovin and colleagues at the Max Planck Institute for Biophysical Chemistry in Goettingen, F.R. Germany.

**Major Findings:** Studies on erythrosin B covalently dye-bound to kidney Na-K ATPase show that the membrane-bound enzyme is highly immobilized compared to other proteins which have been tested, suggesting that the enzyme-ionophore complex is anchored to the cytoskeleton. Two sets of data were collected on Torpedo electric organ microsomal membranes and guinea pig brain membranes. In both cases no changes in lifetime or anisotropy could be detected when  $10^{-8}$  M dye was added to membranes. Addition of salts which activate ATPase had no effect.

**Projected Course:** Dr. Jovin has continued the experimental work with purified membrane bound ATPase from kidney, and has solved many of the problems with the experimental setup. Recent experiments by Silbergeld in this lab have better characterized the requirements for erythrosin B binding to brain membranes, which should greatly reduce signal to background noise in the measurements. Future work will focus not only on the possibility of investigating the binding of free dye to brain fractions but also will attempt to confirm the restricted movement of the structural components of the brain Na pump ATPase. Measurements of erythrosin B interactions with the solubilized receptor will also be made.

## II. Chromaffin granules and chromaffin cells:

- A. Structural arrangement of  $\text{Ca}^{2+}$  in isolated bovine adrenal medullary chromaffin granules (S.J. Morris and J.L. Costa, Clinical Neuropharmacology Branch, NIMH)

**Purpose:** Storage granules like chromaffin granules, synaptic vesicles and platelet granules contain along with chemical neurotransmitters high concentrations of divalent ions, especially calcium. Estimates for  $[\text{Ca}^{2+}]$  in chromaffin granules are as high as 20 mM, and it has been postulated that the calcium is involved in extended structural arrangement of the granule contents. In collaboration with Linda Powers and Brittan Chance at Bell Labs, a project was initiated to examine the possibility of crystalline arrays of calcium by analyzing granule samples for extended atomic fine structure and near edge fine structure of the x-ray excited fluorescence spectra of granule samples.

Methods: Chromaffin granules were prepared from bovine adrenal glands by standard procedures and frozen into special sample holders after the addition of various cryoprotectants. The samples were examined at the Stanford Linear Accelerator Center (SLAC) in Palo Alto, California. The secondary x-ray beam from the SPEAR storage ring is passed through an X-ray monochromator tuned to excite the leading edge of the k-electrons of calcium ions, and then focused onto the liquid-helium cooled specimen. Scattered and fluorescent photons are collected and the frequency spectrum stored for later computer analysis at Bell Labs.

Major Findings: Preliminary results show that the extended atomic fine structure of the granule  $\text{Ca}^{2+}$  is similar to that of platelet dense bodies and both are typical of synthetic model amorphous systems. Further analysis of a newly acquired data set should provide a more quantitative understanding of the structure of the granules.

Projected Course: Depending on the results of the present data set a decision will be made whether or not to pursue this project. As it now stands, little effort is involved since the preps are also utilized for other studies and the material is frozen here and transported to SLAC by Dr. Costa. The effect of manganese and other heavy metal elements known to be taken up by the granules may produce perturbations in the structure of the storage complex and may be detected by this method.

- B. Electronmicrographic studies of the  $\text{Ca}^{2+}$ -promoted fusion of isolated adrenal medullary chromaffin granules. (S.J. Morris with J.D. Robertson, Chairman, Anatomy Department and J.D. Costello, Assoc. Prof., Anatomy Department, Duke University Medical School, Durham, NC).

Purpose: Previous electronmicrographic work has demonstrated that isolated chromaffin granules will aggregate and fuse in the presence of  $\text{Ca}^{2+}$ . The present series of experiments were undertaken first to confirm the earlier work and then extend the observations of suspected intermediate steps in the fusion process which may elucidate the series of molecular rearrangements involved when two tightly apposed biological membranes fuse together.

Methods: Isolated chromaffin granules were exposed to various ionic environments, then either glutaraldehyde fixed, osmium stained and embedded for conventional thin section transmission electronmicroscopy (TEM) or rapidly frozen in liquid nitrogen-cooled liquid propane, fractured and replicated using a system developed by Dr. Costello. The platinum-shadowed replicas were also examined by TEM.

Major Findings: In a series of experiments we have conclusively shown that the granule membranes fuse in the presence of 1 - 5 M mM  $\text{Ca}^{2+}$ . This reaction is independent of ATP and is not inhibited by theophyllin. Extensive analysis of the thin sections on a tilting stage have failed to show any transition figures between the pentalaminar double bilayer and the single fused trilaminar membrane. Earlier freeze fracture work done by the glutaraldehyde fixation, glycerol replacement method showed movement of the membrane-associated particles prior to fusion. This movement has been suggested to be



the result of the preparation method and not to be present when membrane-fusing systems are subjected to quick-freezing followed by fracture. Using Dr. Costello's quick-freeze apparatus, we have shown that the clearing of the particles is present in the quick-frozen preparations. Thus, in this case it is not an artifact of the preparation procedure and may relate to molecular events involved in fusion.

- C. Studies of the kinetics of  $\text{Ca}^{2+}$ -promoted aggregation and fusion of isolated chromaffin granule membranes. (S.J. Morris, D.H. Haynes, Department of Pharmacology, University of Miami Medical School, Miami, FL 33101, and T.C. Suedhof, Department of Neurochemistry, Max Planck Institute for Biophysical Chemistry, Goettingen, F.R. Germany).

Purpose: The calcium promoted aggregation and fusion of isolated chromaffin granules can be modelled as a series of reactions in which the granules first form an encounter complex which is in turn converted to a stable complex of two or more particles. The adhering membranes then undergo a series of reactions eventually leading to their fusion and establishment of patent connections between the core storage spaces of the particles. The first reactions of this scheme have been extensively studied using light scattering readout from a stopped-flow apparatus. We have recently extended these experiments to examine events subsequent to aggregation using fluorescent resonant energy transfer from probes covalently coupled to granule membranes.

Methods: Isolated chromaffin granules were labelled with fluorescent maleimide and mercury acetate derivatives. These reagents couple to free SH-groups. Protein-free lipid extracts of labelled membranes contained virtually no label. Because of the relatively small increases in acceptor fluorescence and the artifactual increase in fluorescence due to particle aggregation, the quenching of donor fluorescence was followed as an unambiguous signal of probe interaction.

Major Findings: (1) If the donor and acceptor were placed on proteins of the same membrane,  $\text{Ca}^{2+}$  increased donor quenching with  $K_m$  of  $\sim 200 \mu\text{M}$ . This  $\text{Ca}^{2+}$  concentration is too low to promote significant aggregation, and the effect is independent of membrane concentration, showing that it must be the result of intramembrane protein clustering or patching. This constitutes the first demonstration of the fluid mosaic nature of a subcellular organelle. (2) If the donor and acceptor labels are placed on separate membranes, mixing in the absence of  $\text{Ca}^{2+}$  produces a slow, essentially zero-order decline in donor fluorescence. This effect can be greatly reduced by extensively washing the membranes and is ascribed to exchange of membrane proteins either through the medium or by granule-granule collisions. If the two sets of labelled membranes are mixed and  $\text{Ca}^{2+}$  added immediately or if the mixture is incubated overnight to allow for complete protein exchange, addition of  $\text{Ca}^{2+}$  produces a protein concentration-dependent increase in donor quenching with a  $K_m$  of  $\sim 2 \text{ mM}$  and rates 5-10 times slower than  $\text{Ca}^{2+}$ -promoted aggregation ( $K_m \sim \text{mM}$ ) measured in parallel. We attribute this fluorescence change to slow rearrangements of membrane components which follow aggregation. (3) We have postulated that granule-granule recognition and aggregation is mediated by proteins which protrude several Å from the membrane p-surface. No component with rates similar to aggregation is seen by the SH-group labelling

method. Since  $\text{Ca}^{2+}$  aggregates labelled or unlabelled granules at the same rate, this implies that if they exist, the protein(s) responsible for granule-granule recognition either contain no free sulphydryl groups or the labelled sites are far enough apart when the proteins interact for no significant Forster energy transfer to occur.

Projected Course: The present set of experiments can be extended to include the effects of various treatments (enzyme digestion, etc) on the energy transfer effects. The basic methodology can also be extended to fluorescent lipid probes which would report changes in the bilayers as aggregation/fusion progresses. Recent synthesis of non-diffusible fluorophores with high quantum yields, combined with a new method developed in this laboratory for resealing granules in the presence of the probes should allow the development of an assay for the fusion of the granule membranes by resealing donor and acceptor molecules into separate populations of granules and recording the mixing of granule contents. This assay will place the probes behind permeability barriers which are not subject to perturbation by substances added to the suspension medium. The effects of inhibitors, drugs, fusion promoters, etc., can then be rapidly assessed in real time (without recourse to time-consuming electronmicroscopy). By this method, the kinetics of each treatment can be analyzed.

- D. Purification of a protein (Synexin) which selectively increases the ability of calcium to aggregate natural and artificial membranes. (J.M.X. Hughes and V.P. Whittaker (Chairman), Department of Neurochemistry, Max Planck Institute for Biophysical Chemistry, Goettingen, F.R. Germany)

Purpose: Synexin is a soluble protein found in relative abundance in the supernatant from homogenized adrenal medullary tissue. The protein specifically increases the ability of calcium to aggregate and in some cases fuse both natural and artificial membranes. Partial purification of the protein has been achieved by several laboratories. This project exploits the ability of the protein to self-aggregate in the presence of calcium to affect a very high degree of purification.

Methods: Crude synexin preparations were prepared as described previously and subjected to repeated cycles of self-aggregation by  $\text{Ca}^{2+}$ .

Major Findings: The method produces a single polypeptide of ~ 46,000 daltons by polyacrylamide gel electrophoresis which has all the calcium specific properties of the crude preparation.

Projected Course: The purified material will be used to produce monoclonal antibodies. These can be used to test for blockage of membrane fusion by toxins (cf project II A and B) as well as disruption of exocytotic release from stimulated primary medullary cell cultures. The purified material can be directly tested for its ability to promote granule fusion, and the kinetics of the promotion studied using the energy transfer techniques outlined above.

PUBLICATIONS:

Fretten, P.M., Morris, S.J., Watts, A. and Marsh.: Lipid-lipid and lipid-protein interactions in chromaffin granule membranes: A spin label study. Biochem. Biophys. Acta 598:247-259, 1980.

Morris, S.J.: Commentary: The structure and stoichiometry of electric ray synaptic vesicles. Neuroscience 5:1509-1516, 1980.

Giompres, P., Morris, S.J. and Whittaker, V.P.: The water spaces in cholinergic synaptic vesicles from Torpedo measured by changes in density induced by penetrating substances. Neuroscience 6:1757-1763, 1981.

Morris, S.J.: Electric ray synaptic vesicle structure and stoichiometry as a function of  $\text{Ca}^{2+}$ -ion chelators. Proceedings of the Third International Conference on Cholinergic Mechanisms, Florence, Italy, March, 1980 (in press).

Morris, S.J., Sudhof, T.C. and Haynes, D.H.: Lipid and protein interactions in calcium promoted aggregation and fusion of chromaffin granule membranes. Biophys. J. 37:1, 1981 (in press).



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02452-01 ODIR																														
PERIOD COVERED      October 1, 1980 to September 30, 1981																																
TITLE OF PROJECT (80 characters or less)  Hormones and Central Neurotransmitter Function																																
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">Robert E. Hruska</td> <td style="width: 25%;">Senior Staff Fellow</td> <td style="width: 10%;">NTS</td> <td style="width: 15%;">NINCDS</td> </tr> <tr> <td>Others:</td> <td>Ellen K. Silbergeld</td> <td>Chief</td> <td>NTS</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>Marc De Ryck</td> <td>Visiting Fellow</td> <td>NTS</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>Roger Weir</td> <td>Guest Worker</td> <td>NTS</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>Karen Pitman</td> <td>Biological Aid</td> <td>NTS</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>Lynn Ludmer</td> <td>Biological Aid</td> <td>NTS</td> <td>NINCDS</td> </tr> </table>			PI:	Robert E. Hruska	Senior Staff Fellow	NTS	NINCDS	Others:	Ellen K. Silbergeld	Chief	NTS	NINCDS		Marc De Ryck	Visiting Fellow	NTS	NINCDS		Roger Weir	Guest Worker	NTS	NINCDS		Karen Pitman	Biological Aid	NTS	NINCDS		Lynn Ludmer	Biological Aid	NTS	NINCDS
PI:	Robert E. Hruska	Senior Staff Fellow	NTS	NINCDS																												
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	Karen Pitman	Biological Aid	NTS	NINCDS																												
	Lynn Ludmer	Biological Aid	NTS	NINCDS																												
COOPERATING UNITS (if any)  None																																
LAB/BRANCH      Office of the Director, IRP																																
SECTION      Neurotoxicology Section																																
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland																																
TOTAL MANYEARS: 3.0	PROFESSIONAL: 1.7	OTHER: 1.3																														
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																																
SUMMARY OF WORK (200 words or less - underline keywords)  This project concerns the effects that hormones, such as estrogen or prolactin, have on neurotransmitter receptors in the central nervous system. We have correlated biochemical changes in striatal dopamine receptors with behavioral changes in stereotypy or catalepsy. Both striatal DA receptor density and behavior of striatal origin are increased by prior estrogen treatment. Prolactin may be the mediator of these effects of estrogen or haloperidol. In addition, several toxins such as PBB have been tested for their estrogenic effects on the striatum. Ergot derivations which act opposite to estrogen in the pituitary have also been examined for their striatal effects. Since aging is associated with hormonal changes, aged rats are used in some studies in the assessment of the effects of hormones. These results may be relevant in <u>neurologic</u> , <u>psychiatric</u> , <u>neuroendocrinologic</u> disorders.																																

## Project Description

### Objectives:

- (1) Investigate the interactions of hormones with central nervous system function.
- (2) Test chemicals and toxins in the environment for hormonal effects on the function of the central nervous system.

### A. Estrogen and Central Dopamine Receptors

Methods: Neurotransmitter receptor binding assays and synaptosomal uptake measurements are used to test the direct actions of hormones on specific receptors isolated from rat brain tissue. These assays are also used on tissue obtained from rats exposed *in vivo* to estrogens.

The behavior of rats is monitored after administration of estrogen. Rats are commonly treated with estrogen by administering 125 micrograms of 17beta-estradiol valerate in sesame oil, subcutaneously, and biochemical or behavioral experiments conducted 6 days later. Two behaviors have been tested: stereotypic behavior induced by DA agonists and cataleptic behavior induced by DA antagonists. Stereotypy is rated by a simple 4-point rating scale.

Catalepsy was rated by means of multiple measures developed by Dr. De Ryck: (1) Akinesia: crouched posture; freezing/shivering reaction; and latency until movement; (2) Bracing reactions; (3) Ptosis; (4) Blepharospasm; (5) Leaning; (6) Clinging; (7) Tonic grasp; (8) Righting; and (9) Tremor. These measures were separately analyzed and converted into a catalepsy index (CI; range = 0-100). Estrogen and control groups were compared once before (saline) and repeatedly up to 7 hours after a suprathreshold dose of 0.25 mg/kg haloperidol. Other estrogen and control groups were similarly tested up to 12 hours after a threshold dose of 0.10 mg/kg haloperidol. Rats were also used as their own controls by running estrogen and control animals once before and 5 hours after saline, followed by observation for 3 hours after the threshold dose of 0.10 mg/kg haloperidol.

Major Findings and Significance: The administration of estrogen to adult male rats significantly increases the density of the striatal dopamine receptors, while not altering their affinity. The biochemical increase is accompanied by a behavioral increase produced by the stimulation of these receptors directly with apomorphine or indirectly by amphetamine. The resultant stereotyped behavior is then measured. Likewise the behavioral response of rotation produced by d-amphetamine in unilaterally lesioned rats (6-hydroxydopamine into the striatum to destroy the dopamine neurons on that side of the brain) is enhanced by estrogen pretreatment.

The biochemical increase follows a slow time course: it takes 3 days to reach significance, is maximally elevated from 4 to 8 days, and returns to normal levels by 11 days. The effect of estrogen on dopamine receptors appears to be specific. Other receptors in the striatum which have been measured do not appear to be changed in either density or affinity. The increase in receptor density is stereospecific since only the gonadally active 17beta-diastereomer of estrogen is active, the 17alpha-diastereomer is inactive. *In vitro* estrogen has no effect on the striatal dopamine receptors.

Our recent behavioral experiments have demonstrated that the neuroleptic haloperidol and the opiate analgesic morphine induce functionally opposite cataleptic immobility states with contrasting effects on postural support

mechanisms. Haloperidol enhances, whereas morphine inhibits, reflex mechanisms underlying static support. Furthermore, these opposite immobility states are characterized by two different and mutually exclusive patterns of rigidity in antagonistic flexor and extensor muscles of the limbs. Haloperidol and morphine also produce distinct neocortical and hippocampal EEG states, which, together with their behavioral correlates, respond in an opposite manner to nociceptive and/or pressure stimuli administered to the skin.

This behavioral analysis of cataleptic states has applied to the interactions between estrogen and dopamine-mediated behaviors. The catalepsy/akinesia induced by neuroleptics, acting as dopamine receptor blockers, has been viewed as one behavioral extreme on a continuum of striatal dopamine function with the other extreme considered to be dopamine agonist-induced stereotyped behaviors. Chronic haloperidol causes tolerance to the catalepsy/akinesia induced by its acute administration. Therefore, we expected that chronic estrogen would attenuate haloperidol-induced catalepsy/akinesia. In fact, the opposite happened: chronic estrogen actually potentiates neuroleptic catalepsy.

Without haloperidol, repeated tests induced a neuroleptic-like catalepsy in 30 percent of drug-free controls. Chronic estrogen reduced such "learned" catalepsy (acute estrogen had no effects). In contrast, after haloperidol treatment, estrogen rats became significantly more cataleptic than controls. After 0.25 mg/kg haloperidol, the catalepsy index of estrogen rats was enhanced by 25 percent over control. Between- and within-animal comparisons showed that estrogen lowered the catalepsy threshold after 0.10 mg/kg haloperidol. These results clearly show that estrogen potentiates haloperidol-induced catalepsy. A simple model of dopamine receptor supersensitivity cannot suffice to explain that chronic estrogen enhances both dopamine agonist- and antagonist-induced behaviors. It is presently unclear whether decreased drug metabolism plays any role in these results.

In striatal tissue from female rats, the density and affinity of dopamine receptors is not different from those in the male rats. However, administration of estrogen to adult female rats has no effect on either the biochemistry of the striatal dopamine receptors or the stereotypy produced by apomorphine. Long-term ovariectomized rats do respond biochemically to estrogen administration with an increase in the density of the striatal dopamine receptors.

Surprisingly, normal female rats have more intense stereotyped behavior after apomorphine administration than normal male rats. This occurs at all phases of the female rats' estrous cycle. Ovariectomized rats display even more intense stereotyped behavior to apomorphine administration than intact female rats. Estrogen administration to ovariectomized rats decreases the behavioral responses to the level of intact female rats, while administration to normal female rats does not change the stereotypy.

The biochemical increase in receptor density and the behavioral increase in stereotypy produced by estrogen treatment in male rats do not occur in hypophysectomized rats. This suggested that a pituitary factor is required as a co-factor or a secondary factor to estrogen to increase the density of the striatal dopamine receptors. However, such a factor is not required for maintenance of the normal receptor density since the surgical removal of the pituitary does not by itself change the striatal dopamine receptors.

The significance of the effect of estrogen on striatal DA receptor function is not known. There are possible interactions with human

neurological binders involving DA. Thus, diseases may be improved or exacerbated by the administration of estrogen or estrogenic compounds.

Projected Course: The importance of the action of estrogen on striatal dopamine receptors will be continued in order to fully assess the changes that occur and their relevance. The striatal dopamine receptors measured by ligand binding are of at least two populations. Localization of the receptors in the striatum will be studied using chemical lesioning techniques. The distribution of the dopamine receptors in the brain and the changes produced by estrogen will also be measured. These experiments will allow the determination of the localization in the brain of the dopamine receptor changes, and the population of striatal receptors affected by estrogen.

Another type of dopamine receptor is linked to adenylate cyclase. These receptors will be investigated by measuring the activation of this enzyme by dopamine and any changes which estrogen or prolactin may cause. Agonists of the dopamine receptor will be tested in receptor binding assays to complement the results obtained with the antagonists, and the information obtained will be relevant to the action of dopamine agonists on striatal receptors.

We would like to investigate whether estrogen also modulates other forms of catalepsy, and in particular, opiate catalepsy, whose behavioral features can be viewed as the opposite of those underlying neuroleptic catalepsy. Morphine-induced rigid immobility is remarkably similar to the behavioral state induced by intracerebral administration of the endogenous opioid beta-endorphin, and this behavioral state has been likened to human catatonia. It is presently unknown whether gonadal hormones may produce sexual differences in endogenous opiate receptor mechanisms. The fact that such mechanisms are clearly involved in affective reactions, including those to pain, make this an important problem. We will therefore determine whether chronic estrogen counteracts or potentiates morphine-induced catalepsy.

## B. Prolactin and Central Dopamine Receptors

Methods: Rats are treated with prolactin by daily injections subcutaneously, or by the use of an osmotic mini-pump which continuously delivers the solution subcutaneously. Injection amounts have varied from 1.0 mg/rat by the daily injection and 120 to 36,000 ng/h by the continuous infusion.

Major Findings and Significance: Since estrogen is known to increase the prolactin secretion of the pituitary, prolactin was investigated as a possible candidate for the co-factor or secondary factor apparently required for the increase in striatal dopamine receptor density. Like estrogen, prolactin has no effect *in vitro* on striatal dopamine receptors. Prolactin administration to adult male rats significantly increases the density of the striatal dopamine receptors and the stereotypy produced by apomorphine administration. Prolactin also increases the density of the striatal dopamine receptors in hypophysectomized rats but a much larger dose was required. Thus, changes in striatal function produced by estrogen may be mediated by prolactin.

The significance of an action of prolactin on the function of dopamine in the central nervous system is under study. Prolactin levels are increased by estrogen, which acts directly on the pituitary and by neuroleptics, which block the pituitary dopamine receptors which normally act to inhibit prolactin release. The relationship of prolactin levels and neurological disorders involving dopamine should be investigated and monitored. The therapeutic



effects of neuroleptics may involve this elevation of prolactin levels, which may lead to increases in the function of the striatal dopaminergic system.

Projected Course: The mechanism of action of prolactin is being investigated. The time course and the specificity of the effects of prolactin will be measured. The observed slow time course in effects of estrogen may be related to its relatively slow action to increase prolactin levels. Prolactin itself may have a rapid time course. If the effect of prolactin on the striatal dopamine receptor is specific, then the usefulness of prolactin as an agent to be monitored is increased.

Like chronic estrogen or haloperidol, chronic high levels of prolactin produce an increase in striatal dopamine receptor density in vivo. If prolactin were directly injected into the neostriatum of rats, would it produce acute neuroleptic-like behavioral effects, i.e., similar to those induced by striatal dopamine receptor blockade, or would it produce acute stereotypic behavioral effects similar to those of DA agonists? Would acute subthreshold dosages of intrastriatal prolactin and of systemic haloperidol summate to neuroleptic catalepsy? Would prolactin, acutely administered to the striatum, counteract dopamine agonist-induced stereotyped behaviors or augment them? The importance of these questions can be justified as follows: (1) even though the presence of prolactin in extrahypothalamic cerebral sites has been documented, its functional role as a presumed neuromodulator in the CNS is unknown; (2) it is unknown whether stimulation of pituitary prolactin release, typically produced by dopamine antagonists, may contribute to their acute neuroleptic effects.

#### C. Chemicals and Toxins

Methods: A variety of other chemicals or poisons are reported to be estrogenic. If this is true, then these agents may affect striatal dopamine receptors by means of their estrogenicity. Examples are the environmental chemicals, PBBs (polybrominated biphenyls) and kepone, and some of the anise oil derivatives. These agents will be tested in vitro and in vivo for the action on central neurotransmitter function and on behavioral functions related to activation or inhibition of the dopamine system.

Major Findings and Significance: PBBs, which are known to be estrogenic, increase the density of the striatal dopamine receptors after in vivo administration. This is consistent with the hypothesis that chemicals which possess estrogen-like activity in peripheral organs such as the uterus may also have estrogenic effects on the central nervous system.

Projected Course: Other estrogenic agents will be tested as they become available for their effects on in vitro receptor binding assays and their ability to increase the density of the striatal dopamine receptors by an estrogenic activity.

#### D. Ergot Derivatives and Central Neurotransmitter Function

Methods: Ergot derivatives are administered in disorders of hyperprolactinemia as well as for the treatment of such neurological disorders as Parkinsonism. Ergot derivatives were tested in vitro for their activity on inhibiting striatal dopamine receptors and other brain receptors. The ergot



derivatives are also tested after in vivo administration by analysing the brain receptors and by monitoring stereotyped behavior.

Major Findings and Significance: Many of the ergot derivatives are potent inhibitors of the striatal dopamine receptors. Some ergot derivatives are potent inhibitors of other neurotransmitter receptors in the brain. Thus the effect of ergot derivatives on the inhibition of prolactin release and their therapeutic and toxic effects in treating neurological disorders must also take into account the direct effects of the compounds on a range of brain receptors.

Projected Course: Several ergot derivatives are being tested further for specific effects on the dopamine receptors and for a possible identification of a derivative that acts only on the pituitary without the direct central receptor effects.

#### E. Aging and Central Neurotransmitter Function

Methods: Aged rats and normal rats (ages: 2, 12, 24 months) are tested for the density of the striatal dopamine receptors and for their response to ergot derivatives and estrogen. Ergot drugs are used therapeutically to treat disease in aged persons and to treat "aging" itself (Hydergine). It is known that in male rats, estrogen levels rise with age while striatal dopamine receptors decrease with age. In female rats, estrogen levels decrease with age. The possible correlation of the known effects of these agents will be tested in our standard assays for in vitro effects and in vivo effects to increase the density of the striatal dopamine receptors.

Major Findings and Significance: Several neurological diseases are associated with aging. However, the relationships between normal processes of aging and the pathophysiology of disease are not known. The density of striatal dopamine receptors are decreased with age. The activity of several ergot derivatives on the ability to inhibit dopamine receptor binding does not appear to change with age. While some of the ergots do not produce stereotypy by themselves, they do augment the stereotyped behavior produced by apomorphine. This suggests that these compounds may have a modulatory role in stereotypy. The ability to increase stereotypy does not appear to depend on age. The significance of these findings to aging is not well investigated. Further work in this area is warranted, especially since some ergots are used therapeutically during aging.

Projected Course: Normal and aged rats will be tested for their response to estrogen to determine if changes occur with aging.

#### PUBLICATIONS:

Hruska, R.E., Ludmer, L.M. and Silbergeld, E.K.: Characterization of the striatal dopamine receptor supersensitivity produced by estrogen treatment of male rats. Neuropharmacology 19:923-926, 1980.

Hruska, R.E. and Silbergeld, E.K.: Review: Inhibition of neurotransmitter receptor binding by ergot derivatives. J. Neurosci. Res. 6:1-11, 1981.

Weir, R.L., Hruska, R.E., and Silbergeld, E.K. Binding of antiparkinsonian ergot derivatives to the dopamine receptor. Psychopharmacology, in press.







ANNUAL REPORT

October 1, 1980 through September 30, 1981

Medical Neurology Branch

National Institute of Neurological and Communicative Disorders and Stroke

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PROJECT REPORTS (Neuromuscular Diseases Section)

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  <div style="text-align: center;">Z01 NS 01189-13 MN</div>
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NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="margin-top: 10px;">           PI: W. King Engel, M.D., Chief, Neuromuscular Diseases Section, NINCDS            OTHER: N. Bojji Reddy, Ph.D., Guest Worker, MNB, NINCDS                      Valerie Askanas, M.D., Ph.D., Associate Neurologist, MNB, NINCDS                      Albert J. Tahmoush, M.D., Clinical Associate, MNB, NINCDS                      Marinos Dalakas, M.D., Asst. Neurologist, MNB, NINCDS                      G. K. Bergely, M.D., NICHD                      P. G. Nelson, M.D., NICHD                      M. Jackson, LB, NINCDS                      B. Joshi, M.D., Clinical Associate, MNB, NINCDS                      Lillian Recant, M.D., VA Hospital, Washington, D.C.         </div>		
COOPERATING UNITS (if any)  <div style="text-align: center;">LB, NINCDS; Laboratory of Developmental Neurobiology, NICHD; VA Hospital, Washington, D.C.</div>		
LAB/BRANCH <div style="text-align: center;">Medical Neurology Branch</div>		
SECTION <div style="text-align: center;">Neuromuscular Diseases Section</div>		
INSTITUTE AND LOCATION <div style="text-align: center;">NINCDS, NIH, Bethesda, MD 20205</div>		
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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 01190-17 MN
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Myasthenia Gravis (MG)		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: W. King Engel, M.D., Chief, Neuromuscular Diseases Section, NINCDS OTHER: Valerie Askanas, M.D., Ph.D., Associate Neurologist, MNB, NINCDS Marinos C. Dalakas, M.D., Assistant Neurologist, MNB, NINCDS John Rose, M.D., Clinical Associate, MNB, NINCDS Charles McIntosh, M.D., Surgery Branch, NHLBI Allen S. Lichter, M.D., RO, COP, DCT, NCI Israel Yaar, M.D., Clinical Associate, MNB, NINCDS Alan Goldstein, Ph.D., George Washington U. Bruce T. Adornato, M.D., Palo Alto Med. Clinic, CA John McClure, Ph.D., George Washington U. Marguerite Foidart, M.D., MNB, NINCDS		
COOPERATING UNITS (if any) Surgery Branch, NHLBI; RO, COP, DCT, NCI; George Washington University, Washington, D.C.; Palo Alto Medical Clinic, CA		
LAB/BRANCH Medical Neurology Branch		
SECTION Neuromuscular Diseases Section		
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PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Myopathies		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <div style="display: flex; justify-content: space-between;"> <div style="width: 10%;">             PIs:               OTHERS:           </div> <div style="width: 85%;">             W. King Engel, M.D., Chief, NMB, MNB, NINCDS              Valerie Askanas, M.D., Ph.D., Associate Neurologist, NMB, MNB, NINCDS              N. B. Reddy, Ph.D., MNB, NINCDS              B. Lavenstein, M.D., MNB, NINCDS              A. Tahmoush, M.D., MNB, NINCDS              M. Foidart, M.D., MNB, NINCDS              M. Dalakas, M.D., MNB, NINCDS              B. Joshi, M.D., MNB, NINCDS              I. Yaar, M.D., MNB, NINCDS              B. Blumenkopf, M.D., MNB, NINCDS              A. Galdi, M.D., MNB, NINCDS              M. Zweig, M.D., CP, CC           </div> </div> <div style="text-align: right; margin-top: 10px;">(Continued below)</div>		
COOPERATING UNITS (if any) CP, CC, NIH; NEI; NICHD; NHLBI; Institut de Pathologie Moleculaire, Paris; NIAID; NMNC; UCSF; VA Hosp., Boston, MA and Madison, WI; IDB, NINCDS; Johns Hopkins University, MD; Palo Alto Medical Clinic, CA; Downstate Medical Center, NY; University of Florida.		
LAB/BRANCH Medical Neurology Branch		
SECTION Neuromuscular Diseases Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD 20205		
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SUMMARY OF WORK (200 words or less - underline keywords)  This project has been terminated.  <hr/> Other professional personnel continued:  A. Lichter, M.D., RO, NCI A. Shug, Ph.D., VA Hospital, Madison, WI J. Avigan, Ph.D., NHLBI D. Valle, M.D., Johns Hopkins, Baltimore, MD M. Kaiser-Kupfer, M.D., NEI B. T. Adornato, M.D., Palo Alto Med. Clinic, CA R. W. Kula, M.D., Downstate Med. Center, NY K. Zis, M.D., MNB, NINCDS		
(Continued)		

## Other professional personnel continued:

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U. Muller-Eberhardt, M.D., UCSD  
P. G. Nelson, M.D., NICHD  
G. K. Bergey, M.D., NICHD  
R. F. Bonner, BEI, R  
P. D. Bowen, IR, TD, NHLBI  
B. R. Line, M.D., NM, CC  
H. M. Fales, Ph.D., NHLBI  
L. Prockop, M.D., U. Florida  
T. Sunder, M.D., NNMC  
S. W. Hosea, M.D., LCI, NIAID



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PERIOD COVERED <p style="text-align: center;">October 1, 1980 through September 30, 1981</p>		
TITLE OF PROJECT (60 characters or less) <p style="text-align: center;">Amyotrophic Lateral Sclerosis (ALS), Other Lower Motor Neuron Diseases, and Peripheral Neuropathies</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <div style="display: flex;"> <div style="flex: 1;">           PI: OTHER:         </div> <div style="flex: 4;">           W. King Engel, M.D., Chief, NMD Section, MNB, NINCDS            V. Askanas, M.D., Associate Neurologist, MN, NINCDS            M. Dalakas, M.D., Clinical Associate, MNB, NINCDS            H. B. Levy, M.D., Laboratory of Viral Diseases, NIAID            I. Yaar, M.D., Clinical Associate, MNB, NINCDS            P. G. Bernad, M.D., Clinical Associate, MNB, NINCDS            J. E. Kurent, M.D., Clinical Associate, MNB, NINCDS            B. Joshi, M.D., Clinical Associate, MNB, NINCDS            B. R. Brooks, M.D., Johns Hopkins            B. T. Adornato, M.D., Palo Alto Medical Clinic            J. B. Nutt, M.D., U. Oregon            J. W. Griffin, M.D., Johns Hopkins         </div> </div> <p style="text-align: right;">(Continued below)</p>		
COOPERATING UNITS (if any) LVD, NIAID; LCS, NIMH; LEP, NIAMDD; IDB, NINCDS; CC, NIH; Johns Hopkins University; WRAMC; VA Hospital, Washington, D.C.; D, DCBD, NCI; Palo Alto Medical Clinic; University of Oregon; PO, COP, DCT, NCI.		
LAB/BRANCH Medical Neurology Branch		
SECTION Neuromuscular Diseases		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD 20205		
TOTAL MANYEARS: <p style="text-align: center;">5.2</p>	PROFESSIONAL: <p style="text-align: center;">3.7</p>	OTHER: <p style="text-align: center;">1.5</p>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS         </div> <div> <input checked="" type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input type="checkbox"/> (c) NEITHER         </div> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div> <input checked="" type="checkbox"/> (a1) MINORS         </div> <div> <input type="checkbox"/> (a2) INTERVIEWS         </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords)  <p>This project has been terminated.</p> <p>Other professional personnel continued:</p> <p style="margin-left: 40px;">             H. R. Gralnick, M.D., CC, NIH              S. A. Houff, M.D., IDB, NINCDS              S. L. Sever, M.D., IDB, NINCDS              Lillian Recant, M.D., VA Hospital, Washington, D.C.              S. Bhatthema, Ph.D., VA Hospital, Washington, D.C.              M. A. Flaum, M.D., CC, NIH              D. L. Madden, D.V.M., Ph.D., IDB, NINCDS              G. G. Glenner, M.D., LEP, NIAMDD           </p>		

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# ANNUAL REPORT

October 1, 1980 through September 30, 1981

## Surgical Neurology Branch

National Institute of Neurological and Communicative Disorders and Stroke

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## ANNUAL REPORT

October 1, 1980 through September 30, 1981

Surgical Neurology Branch, IRP

National Institute of Neurological and Communicative  
Disorders and Stroke

Paul L. Kornblith, M.D., Chief

### Summary of Studies in the Surgical Neurology Branch

This annual report is the third of the Surgical Neurology Branch beginning October 1, 1980 under the current leadership of Dr. Paul Kornblith. Over the past year both the research and clinical programs of the Branch have become fully operative and productive. The electron microscopic and image analysis facilities of the Branch have now been completed and the programs dependent on these set into motion. The tissue culture facilities have seen high utilization and their expansion is continuing. Tumors from our NIH patients and from other centers in the U.S. are under detailed study. Immunological chemotherapy and basic cell biological studies of human gliomas and other CNS tumors are carried out with these tumor cell lines. New research programs in cellular immunology, humoral immunology and antigenic purification for gliomas have been set up and are now fully active and productive.

Over the period of this report, between ninety and one hundred surgical cases have been done, which have provided new tumor material for study. Major upgrading of the surgical facilities has been ongoing and has included the installation of an argon laser. Metabolic studies of patients with brain tumors have been initiated as well. Initial studies with the positron emission tomographic scanner have shown a relationship between glucose uptake and degree of tumor growth. A new microsurgical laboratory has been developed to prepare our branch for microvascular and microsurgical approaches to CNS tumors and vascular lesions.

The primary areas of our research activities have included:

1. Biological, immunological and chemotherapeutic studies in human brain tumors.
2. Biological and immunological factors in peripheral nerve injury, neoplasia and regeneration.
3. Biological studies of human pituitary tumors.
4. Neurodiagnostic studies including the PECT scan.
5. Neurophysiological studies.

Clinical protocols are now approved and underway. These are:

1. Evaluation of Biological, Immunological and Chemotherapeutic Parameters in Brain Tumor Patients (79-N-89)

2. Immunotherapy of Malignant Brain Tumors (79-N-133)
  3. Biological Studies of Human Pituitary Tumors (79-N-151)
  4. Evaluation of Biological and Immunological Parameters in Peripheral Nerve Regeneration (80-N-06)
  5.  $^{18}\text{F}$ -2-Fluoro-2-deoxy-D-Glucose (FDG) Positron Emission Computed Tomography (PECT) in Typing of Cerebral Gliomas (80-N-36)
  6.  $^{18}\text{F}$ -2-Fluoro-2-deoxy-D-Glucose (FDG) Positron Emission Computed Tomography (PECT) in Epilepsy (8-N-58)
  7. Phase II Trial of AZQ in Patients with Malignant Glioma and Metastatic Brain Tumors (joint with NCI)
  8. A Phase I Study of Bromodeoxyuridine (NSC 38297) Given by Peripheral Venous Infusions (joint with NCI)
1. BIOLOGICAL, IMMUNOLOGICAL AND CHEMOTHERAPUETIC STUDIES IN HUMAN BRAIN TUMORS

A. Biological Characterization and Neuropathological Studies

In order to determine the ways in which human brain tumors will behave in a given patient, it is necessary to have cellular models of their biological activity. Such cellular models are provided by the tissue culture system. In this system it is possible to grow approximately 90% of human brain tumors in an environment outside of the human body. Utilizing this approach it has been possible to show that certain characteristics of cultured human brain tumor cells not only parallel those of the cells in a patient but also provide the opportunity to add therapeutically relevant information to the planning of optimal therapy and the prediction of the way in which a tumor will grow in a given patient. This type of work has two major areas. First is the area of the prediction of the behavior of tumors which are known to be malignant. Here the major question is how malignant a given tumor will be. Secondly, in certain tumors, which by and large are benign or nonmalignant in their growth, there are occasional instances in which tumors do grow in a malignant fashion. In the second category, the question is how to pick out ahead of time those tumors which behave in a malignant or invasive fashion. These are the two primary goals of the program in the study of tumor biology. There are, in addition, several secondary goals. These include: studies of the basic biologic mechanisms of tumor growth and the similarities and differences of this tumor growth to the growth of normal cells. In order to achieve successful evaluation of all of these primary and secondary goals, a tissue culture laboratory has been established in the SNB for the study of human brain tumors.

Of major importance for these studies has been the building of our electron microscope laboratory (under Dr. Barry Smith's direction) which now includes two transmission electron microscopes, a scanning microscope, x-ray spectroscopy, and image analysis capabilities. These facilities are in operation now. Further testing and adjustment of some of the more technologically sophisticated x-ray and image analysis equipment continues but the primary electron microscope functions are fully active. General EM transmission characterization of 40 glioma lines is being carried out in addition to studies of special surface staining properties. Scanning and TEM microscopy of isolated tumor cells and tumor cells interacting with lymphocytes has allowed visualization of tumor to immune cell interaction.

Dr. Maurice Gately, a cellular immunologist who set up the SNB cellular immunology laboratories, continues a major study of specific and non-specific lymphocyte-mediated tumor cell cytotoxicity. Additionally, Dr. Nobuyuki Shitara, a Visiting Scientist, has made further progress in the study of techniques of flow cytometric DNA analysis that have been applied to the characterization of approximately 20 glioma cell populations. He has also studied coupled desensitization of beta-adrenergic receptor-adenylate cyclase and found increased glucose uptake in C6 gliomas in vitro. Cells cultured from human and rat glioma released proteins into the culture medium. These proteins may be secreted or shed from the cell surface. Dr. Paul McKeever, the SNB neuropathologist, has also been very productive over the period of this Annual Report. Further characterization of the protein products of cells cultured from gliomas has included kinetic studies of protein release, studies to gauge cytolytic factors and the shedding of vesicles in protein release, protein glycosylation studies and attempts to specifically identify the nature of these release proteins. In light of the observations made during the characterization of human lines, the C6 clonal glial cell line was studied as a positive central line in addition to the human line. Pulse-chase experiments with labeled amino acid protein precursors show an initial lag phase, a sharp rise in protein release, followed by a cessation of release. These kinetics resemble secretory events in other cell types. Trypan blue exclusion, lactic dehydrogenase and freezing-thawing experiments showed that the cells actively release a set of proteins different than those released by cytolysis and that cytolysis was negligible under standard conditions of biosynthetic protein labeling. Ultracentrifugation and millipore filtration experiments show a major portion of the released proteins to be different than those associated with membrane vesicles of the type which carry phosphoesterhydrolases and a 5' nucleotidases. Some released proteins are glycosylated, as would be expected, if they were processed through the Golgi apparatus. The explanation consistent with this group of data is that cells cultured from gliomas secrete a defined set of proteins.

Dr. McKeever has also provided neuropathological classification of SNB tumors both in operating room specimens and in subsequent tissue culture. Clinical neuropathology has been improved at the

NIH and at present, three neurosurgery residents and five pathology residents are receiving neuropathology instruction. Plans to formalize a neuropathology program are underway. Consultations have increased from zero to about six per month. An effective working relationship has been established with the laboratory of Pathology at the Clinical Center and with the Neuropathology Division of the Armed Forces Institute of Pathology, extending histologic and diagnostic capabilities and increasing the variety of material available for analysis.

Frozen section diagnosis has been improved with the use of immunocytochemistry to localize tumor antigens, specifically glial fibrillary antigen and fibronectin. Recognition of glial fibrillary antigen in neoplastic cells allows immediate differentiation between fibrillary gliomas and schwannomas or meningiomas. Staining for fibronectin offers similar power of discrimination by localizing meningotheial cells, Schwann cells and fibroblasts.

Dr. McKeever, together with Dr. Barry Smith and electron microscopy support staff, has proceeded with an in-depth ultrastructural characterization of available glioma lines including intercellular junctions, glial filaments, surface membrane parameters and nuclear morphology. Since precise characterization of the cells from tumors and in culture is so critical to all the work for the SNB, the establishment of this capability is a major step forward for the SNB. Dr. Bibie Chronwall, a Visiting Fellow who joined the SNB in February, has been using immunohistochemical methods (peroxidase and fluorescein) to develop rapid diagnostic methods for brain tumors. These same techniques are being used to define tumor cells under various conditions in tissue culture.

Each of these specific advances in biological characterization fit into our overall ongoing program of glioma cell analysis. These ongoing studies include analyses of the rate of growth of individual as well as populations of tumor cells; the ability of tumor cells to grow under stressful conditions such as in soft agar or in low serum concentrations; their ability to invade neighboring tissue; their chromosomal pattern and abnormalities; and their growth and malignancy in animal hosts. This latter technique is perhaps the most crucial in determining whether an individual human tumor can reproduce via tissue culture a tumor in the in vivo situation.

These studies of tumor malignancy are supplemented by studies of tumor characterization in which tumor cell populations are characterized as to their cell or cells of origin. It is extremely important in deciding whether or not a given tumor will behave in a malignant or in a benign fashion, relatively speaking, to determine what type of cell predominates. Frequently, human brain tumors are of mixed cell origin or at least mixed cell origin as regards the degree of malignancy of the cell comprising the tumor mass. Our tumor characterization includes detailed studies of cellular ultrastructure membrane and



cytoplasmic biophysical properties and biochemical properties, such as S-100 protein, the enzyme cortisol acetyltransferase and glial fibrillary acidic protein (See above). In addition, studies of myelin basic protein and cerebral gangliosides may be of importance. Such studies are proceeding on the tumor cell lines under evaluation.

From all these biological studies it appears possible (as we have reported previously) to determine the degree of malignancy and to characterize the cell of origin of a given tumor. This data can be useful in determining the prognosis of the individual patient and also in the planning of individualized, optimal therapy. For example, it is possible to assess the likely responsiveness to radiation therapy from the type of kinetic growth pattern of a particular tumor. The most rapidly growing tumors in tissue culture are the most sensitive to radiation. Likelihood and rapidity of recurrence may also be determined.

In order to understand the metabolic processes basic to human glial cells, we have examined several aspects of the glycolysis of cultured astrocytomas grades III and IV including glucose uptake, lactate and pyruvate efflux, glutamate and glutamine uptake and glycogen levels. These observations are relevant not only to the growth of glial tumors in culture but also to the correlation of clinically derived data from the PECT scan.

#### B. Immunological Studies

It is highly likely that immunotherapy will be an important part of brain tumor therapy in the near future. With this in mind, development of a systematic approach to the study of the immunological aspects of brain tumors both with respect to the tumor and the host has been undertaken. This includes the cellular as well as the humoral facets of immunological interaction. We have been extremely fortunate to have the contributions of Dr. Eugene Quindlen, a skilled humoral immunologist, as well as Dr. Maurice Gately, an excellent cellular immunologist. We thus have special expertise in the two crucial areas of immunological interaction in human tumors. We have made further progress in our laboratory for the study of immunology, which includes extensive technology for the separation of immunoglobulins, for the characterization of immune globulin and antigen activity and for the characterization of cellular subpopulations of lymphocytes, as well as the study of their interaction with brain tumor target cells. Further, we now have in force an immunotherapy protocol for glioma patients which is among the first three in the world.

The approach which we are using is to study the immune response in tissue culture utilizing individual patient's tumor cells as targets. The cultured tumor lines enable us to study the interaction in a very direct way with humoral factors and cellular factors studied either separately or in combination.

The humoral immunological techniques have a potential direct diagnostic value. The detection of antibody activity in the serum of patients harboring brain tumors can be made in over 80 percent of such patients.

A simple diagnostic test of immune response in individual patients for telling whether they may or may not have a brain tumor is thus a practical reality. More needs to be determined about the specificity of the response. To date we know that it is present in "normals" at the level of only 9 percent. Patients with metastatic tumors show a 30-40 percent positive response.

Autologous cytotoxic antibody was detected in 75 percent of astrocytoma patients, compared with 17 percent of glioblastoma cases; the difference being highly significant. When the same sera were tested allogeneically on a common glioma culture, there was no such difference. A strong correlation was also found between seropositivity and survival and with age. Tumor grade and age, along with functional status (Karnofsky scale) at diagnosis are the best prognostic factors for survival of glioma patients.

The autologous cytotoxic humoral response is the first instance of an immunological response which, when taken in isolation, can also predict survival in adult glioma patients. This potential has been previously sought, but not confirmed, in the following tests of general immune responsiveness: immunoglobulin and lymphocyte serum levels, lymphocyte subpopulation assays, recall antigen skin testing and serum blocking activity. The present evidence does not suggest that cytotoxic antibody assay offers additional advantages over clinical and histological predictive factors. However, in analyzing the lower grade astrocytoma cases, seropositivity correlated with increased survival, but insufficient numbers were available to permit statistical significance.

Tissue culture target glioma cells are crucial for our new immunotherapy program, which can only be effective if there is an understanding of baseline immunological responses. We have looked for autologous cytotoxic antibodies by means of a complement-dependent microcytotoxicity assay in the serum of over thirty patients. The lower grades of astrocytomas, Grades I-III have a consistently higher rate of positive responses than do the Grade IV's. It also seems that the presence of cytotoxic antibody correlates with longer survival. Autologous fibroblasts were also tested in fifteen patients. All fibroblasts were negative for cytotoxic antibody, supporting the fact that there is some degree of tumor specificity for this serological assay. Our immunotherapy program is presently dealing with the question of growing and maintaining cells in autologous human serum without addition of fetal bovine serum. We have successfully injected 3 primates with irradiated human tumor cells. Three patients have been given their own cells to enhance their immune responses.

Investigation of different variables which may modulate immunological competence may include calcium, the steric relationship of the tumor itself and immuno-modifiers such as cAMP, Con A, anesthetics and Atromid-S. If the mechanisms of these variables can be understood, it may be possible to modify immune response in vivo by modifying their interaction.

Antiglioma antibodies in sera from families where one or more members have neurofibromatosis has also been studied. Preliminary results are somewhat encouraging and show a possible positive correlation between presence of antibodies and clinical disease. Further investigation will be necessary to determine how precise this correlation is and whether there is predictive value in our assay for siblings who do not yet show clinical signs of neurofibromatosis.

Major goals of our work include development of our immunotherapy program and of our definition and modulation of the humoral and cellular immune response.

A major effort has been expended in the area of cellular immunology of human brain tumors. The long term objective of this project is to provide a basis for the rational immunotherapy of brain tumors by developing information regarding the interactions between human brain tumors and the cellular immune system.

In this work, freshly isolated peripheral blood lymphocytes from each of 6 glioma patients failed to cause significant lysis of autologous glioma cells even though these patients, at least early in the course of their disease, displayed little or no depression of lymphocyte function as measured by the ability of their lymphocytes to respond to mitogens and in mixed lymphocyte cultures. Studies on the ability of human glioma cells to elicit cytolytic lymphocyte responses in vitro may help to explain the failure of patients to make cytolytic lymphocyte responses to their own tumor in vivo. These studies have indicated that human glioma cells possess at least three mechanisms by which they may escape lymphocyte-mediated destruction: (1) low immunogenicity, perhaps related to a defect in their ability to stimulate helper T cells, (2) the secretion of immunosuppressive factor(s), and (3) the production of a protective mucopolysaccharide coat which impeded contact between lymphocytes and the glioma cells.

### C. Chemotherapy Studies

At the present time the major therapeutic modality being explored to alter the prognosis in human brain tumors is that of chemotherapy. The most effective forms of chemotherapy - the nitrosourea compounds - are effective in only 50 percent of patients. Therefore, it is important to determine which patients will respond to which agent. We have developed a system, again using our tissue culture approaches, whereby the individual patient response can be determined. Using this response we have been able to show that there is a direct correlation between individual patient response to a given agent in their tissue cultures and their response clinically. This has necessitated the use of CT scan follow-up, as well as clinical evaluation on a serial basis. As shown in our recent paper in Cancer, we have evaluated over 50 patients, only 14 of which met both in vitro and in vivo criteria for study. Six of nine cell lines (67%) responding to BCNU in vitro were shown to correlate with tumor sensitivity clinically, i.e., tumor size decreased in these

patients. Five of five patients were shown neither to be sensitive in vitro nor in vivo (100%). Currently, an additional series of patients is being evaluated for conformance to study criteria.

The observation that there is a correlation enables us to do more extensive studies in determining why there are or are not specific responders to chemotherapeutic agents. These studies of specific responders and nonresponders have included differences in membrane characteristics such as surface coating and membrane fluidity; in state of differentiation; in oxidative metabolism and in protein synthesis. Agents used for these studies include Concanavalin A, dibucaine, s-adenosyl-L-methionine, and puromycin. Collaborative studies of DNA strand break repair have thus far revealed that drug action is not limited to one mechanism alone. Preliminary study of the nitrosoureas indicates that cellular sulfhydryl concentration may be important. Ongoing studies are examining this area in greater detail. From these more extensive and sophisticated studies we may be able to learn what makes a cell or a given tumor respond or not respond to chemotherapy.

We are presently developing our model system for a more detailed and thorough study of how the drug interaction with the more commonly used agents (e.g., nitrosoureas, vincristine, procarbazine, methotrexate), as well as some of the new agents, can be evaluated on a patient by patient basis with the hope of developing a rational prospective chemotherapeutic plan and with the hope that agents or factors enhancing the response may be developed. Both cytotoxic and cell differentiation agents (e.g., cyclic adenosine monophosphate, dimethylformamide, DMSO, and hexamethylene bis acetamide) are being explored for their potential interactions leading to enhanced killing of neoplastic cells.

We are currently using a modification of the microcytotoxicity assay to test new chemotherapeutic agents for the National Cancer Institute. Many of these agents are not soluble in aqueous media, presenting the problem of solvent toxicity. The new solid-phase drug delivery system permits the evaluation of agents (e.g., CCNU, PCNU, AZQ, spirohydantoin and rapamycin) that would otherwise be impractical in the microtiter system. Preliminary study has shown the effectiveness of some of these drugs as chemotherapeutic agents. In our studies of AZQ it was tested on 23 cell lines, using the solid phase chemotherapy assay. Varying dose response curves were seen with the different cell lines. These results are now being correlated with the results of our clinical phase II trial of AZQ in our astrocytoma patients.

As part of an exploration of the effects of the various components of standard glioma therapy, the effects of phenytoin (used as a prophylactic anticonvulsant after glioma surgery) was examined and an inhibition of the growth of at least 50 percent of glial brain tumors was found. We have previously published human brain tumor data on this inhibition and have now documented that findings in two rat glioma tumor models (C6 and RT9). Acute and chronic survival experiments using the RT9 (in a subcutaneous tumor model) have been conducted.



Consideration of dilantin metabolism kinetics has resulted in studies of staggered delivery schedules and the use of subcutaneously-implanted osmotic pumps.

In vitro experimentation with cultured TR 9 and C6 tumor cells is being conducted with the microcytotoxicity assay. These experiments confirm our previous studies of murine tumor sensitivity to dilantin. Further work to elucidate the basis of the tumor growth-inhibitory effect of phenytoin is proceeding.

## 2. BIOLOGICAL AND IMMUNOLOGICAL FACTORS IN PERIPHERAL NERVE INJURY, NEOPLASIA AND REGENERATION

As described in the previous annual report, a vein grafting technique for rat sciatic nerve has been developed and perfected and is continuing to serve as a model system for the study of molecular and cellular factors in neuronal regeneration after injury. The vein graft serves to provide a chamber into which various molecular factors such as collagen or growth factors (epidermal growth factor, nerve growth factor, fibronectin, etc.) or cells such as cultured fibroblasts, Schwann cells, central glial or glioma cells can be introduced to study their effects on axonal regrowth through the graft. Some 60 long- and short-term experiments have been completed and are being analyzed by a variety of light and electron microscopic as well as image analysis techniques. Further studies of the influence of various lectins on the regrowth of injured axons and their interaction with target tissues have been carried out in an effort to validate the hypothesis that glycoproteins are a critical element.

Human peripheral nerve cultures derived from several patients with neurofibromatosis have been established. Of particular interest have been the growth control mechanisms of these abnormal but non-malignant cells.

Finally, two patients have undergone resection of neurons resulting from failed or abnormal nerve regeneration under the Surgical Neurology Branch nerve injury protocol. These neuromas have been studied in tissue culture and in the light and electron microscopes to determine the patterns of biological response to nerve injury that result in failed regeneration. The signal leading to Schwann cell and fibroblast proliferation after axonal injury is of special interest and a major effort of this project will be to identify that "signal".

## 3. BIOLOGICAL STUDIES OF HUMAN PITUITARY TUMORS

The secretion of hormones by normal and neoplastic anterior pituitary cells has been an ongoing effort of this laboratory for several years. A surprising finding has been that certain pituitary tumor cells in tissue culture secrete not only the hormone which they have classically been known to produce clinically but also a range



of other hormones. The explanation for this "dedifferentiated" behavior in molecular terms should provide insight into the abnormalities of the growth regulatory mechanisms of these neoplastic cells as well as to the clinical endocrinological picture they may present. In addition, studies of the variations of different pituitary tumor cell populations should be useful in elucidating the reasons why some tumors recur and/or are invasive whereas others do not.

Secreted hormone profiles over periods of time ranging from 1 week to several months have been obtained on some 10 human pituitary lines removed at surgery and grown in tissue culture. Hormones included in the assay have included ACTH, GH, PRL, LH, and FSH. These assays have proven to have multiple values including the identification of particular tumor types when the pathology is not conclusive, i.e., for the diagnosis of tumor types.

The pattern of hormone secretion observed in these pituitary lines has confirmed previous results in that many of the lines produce several hormones in addition to the one the pathology would have predicted. One line, however, which could not be identified on the basis of standard pituitary pathology was shown by hormonal assay to be a clear prolactinoma and not to secrete other hormones. Follow up of the patterns of hormonal secretion of these cell lines in culture over several weeks has shown a fall-off in secreted hormone production over this period. This decrease in hormonal production can, however, be partially reversed by administration of dibutyryl cyclic AMP. It seems quite possible that analysis of pituitary tumor hormone secretion patterns will lead to a much more precise and meaningful diagnostic classification of pituitary tumors.

Aside from the diagnostic classification of pituitary tumors, the pattern of hormone secretion yields the opportunity to study the regulation of phenotypic expression in neoplastic pituitary cells. This, of course, has implications for determining both the state of differentiation of each pituitary (and hence its malignant potential) as well as the nature of the abnormal growth control mechanisms. This area has been pursued over the past year and will remain the subject of major continued effort of the malignant biological potential of a given tumor line (and hence prediction of clinical course) as well as the development of biological means of controlling abnormal pituitary cell growth in patients.

The third major area of interest for pituitary cells in culture is the study of the mechanisms and regulation of the secretion of pituitary hormones. The *in vitro* system is especially useful for the analysis of these cellular mechanisms. In studies completed to date dibutyryl cyclic AMP appears to have a regulatory role in the synthesis and/or secretion of luteinizing hormone and follicle stimulating hormone but not ACTH, GH, or prolactin to any significant degree. Receptor-coupled dopaminergic regulation of prolactin secretion is also under study utilizing bromocriptine. With the recent arrival of Dr. Craig Cummins,

who is an expert in receptor biochemistry, this area will be the subject of increased effort in the coming year.

#### 4. NEURODIAGNOSTIC STUDIES INCLUDING THE PECT SCAN

##### RESEARCH IN THE "NEURORADIOLOGY AND COMPUTED TOMOGRAPHY SECTION"

The Neuroradiology and Computed Tomography Section has spent the bulk of its research efforts concentrating on computed tomography (CT) in both its transmission and emission (Positron Emission Tomography) modalities.

Positron Emission Computed Tomography (PECT) represents a totally new approach to the understanding of the physiopathology of many neurological diseases. An innovation of PECT is that it provides physiologic information not available with any other imaging procedure. We can now obtain pictorial data. (E.G., coronal images or axial transverse images of the brain) in addition to dynamic functional data (this would include measurements of the storage, degradation and turnover of tagged metabolites, and follow-through of the movement of the CSF in the deep CSF intracranial cavities).

During the past year, construction of the new, high resolution, NIH-built PECT-scanner for head and animal studies (the Neuro-PET) has become very advanced. Phantom testing should begin in the next few weeks and patient scanning in several months.

Three clinical protocols for the PECT scanner have been instituted. Over 20 patients with gliomas have now been studied with <sup>18</sup>F-deoxy-glucose, and more than 10 patients with epilepsy have received similar treatment. A protocol dealing with Alzheimer's disease has similarly been instituted.

Computed Tomography (CT) in its transmission modality represents the other main research area of the Neuroradiology and Computed Tomography Section.

Our work involves continuing clinical-animal/experimental research projects in transmission CT. This includes studies of demyelinating, degenerative and trophic processes of the brain, brain edema, hydrocephalus, postradiation, cerebral necrosis, diseases of the spine and the spinal cord, surgically correctable lesions in young patients affected by chronic epilepsy, attempts at tissue characterization of normal and tumoral cerebral tissue, and an experimental glioma model in primates.

The physics projects include the analysis of aliasing effects and development of methods for their elimination; improved dual-energy CT scanning using both a split-detector and a dual kVp method; feasibility tests for a new type of CT device using protons instead of x-rays; phantom studies for the evaluation of artifacts and calibration of CT machines.

Experimental spinal cord angiography in the rhesus monkey has augmented our knowledge of the blood supply of the spinal cord both in physiological and pathological conditions. Lately this technique has been employed as a basis for attempts at experimental surgical revascularization of the cord in primates. As part of this study microsurgical revascularization of the spinal cord is now being carried out in monkeys.

Radionuclide ventriculography and cisternography are diagnostic tools allowing the dynamic and morphologic study of the cerebrospinal fluid pathways more completely than was ever possible with any other diagnostic test.

The addition of positron emission computed tomography to our diagnostic armamentaria should significantly improve the information content of our cisternograms and our radionuclide ventriculograms.

Selective radiography (radiographic) of the spinal cord is a diagnostic technique which has been most informative in cases of tumor, arteriovenous malformation, trauma, obstructive vascular disease, and post-radiation damage of the spinal cord.

Radioisotope angiography of the spinal cord offers distinct advantages as a method of screening, and may give information not available by any other diagnostic test in certain kinds of intraspinal pathology.

Our preliminary experience with computed tomography of the spine after injection of contrast medium shows that this methodology is helpful in the evaluation of certain vascular lesions of the spinal cord.

## 5. NEUROPHYSIOLOGICAL STUDIES

Under the direction of Dr. Choh-Luh Li, investigations of the neurophysiological mechanisms of pain have been carried out. Over the course of the year two new senior scientific personnel have been added to augment these studies: Dr. Chang Hsiang-Tung of the Peoples Republic of China and Dr. Takekane Yamaguchi from the University of Tokyo. Emphasis has continued to be on pain mechanisms using the cat vagus nerve model. The left vagus nerve and right sural nerve are utilized and brain stem recordings have been made in the ganglion nodosum, nucleus tractus solitarius and nucleus parafascicularis. Responses recorded from cells in the nucleus tractus solitarius have been shown to be characteristic in their latency and discharge patterns. Stimulation of the sural nerve seems to change these neuronal response characteristics and so presents a very valuable model for the neurophysiological interaction and modulation of visceral pain. Current efforts are devoted to 1) more detailed recordings in each of the above-mentioned areas; 2) horseradish peroxidase tracing of vagal nerve fibers and axons of the nucleus tractus solitarius and 3) extracellular potential recordings as well as iontophoretic application of neurotransmitter substances or candidates to these target areas.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> <b>INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER Z01 NS 02367-03-SN
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less) Biological, Immunological and Chemotherapeutic Studies of Human Brain Tumors		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Other:	Paul L. Kornblith Barry H. Smith Eugene A. Quindlen Maurice K. Gately Paul E. McKeever Moshe Glaser Nobuyuki Shitara Bibie Chronwall Craig Cummins Yoshio Moriya	Chief Deputy Chief Senior Staff Fellow Senior Staff Fellow Medical Officer Expert Visiting Scientist Visiting Fellow Staff Fellow Visiting Fellow
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COOPERATING UNITS (if any) Division of Radiation Therapy, NCI		
LAB/BRANCH Surgical Neurology Branch		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3.0	PROFESSIONAL: 3.0	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Human brain tumors are evaluated in a <u>tissue culture</u> environment as to their basic <u>biological</u> behavior, their response to <u>chemotherapeutic</u> agents and the detailed <u>immunological</u> interactions between the host and the tumor. A primary goal is to improve the <u>therapy</u> of patients by understanding the basic <u>cellular biology</u> of malignant human brain tumors.  SNB has continued expansion of the biological characterization program with the inclusion of karyotyping, glial fibrillary acid protein and fibronectin assays, DNA repair, adrenergic and other receptor assays, ganglioside and glycoprotein assays, cloning techniques, in-depth neuropathological studies, and automatic image analysis; utilized both aqueous and surface chemotherapy assays to test several new potential antglioma agents and initiation of clinical trials with one such agent, AZQ; defined the basis of cellular sensitivity or resistance to nitrosoureas; characterized the humoral cellular immunological response to gliomas; and initiated correlative cellular and PECT scan glucose metabolic studies.		

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## PROJECT DESCRIPTION:

I. **OBJECTIVES:** There are three primary objectives of this research program. The first objective is to determine how increased understanding of the cellular biology of human brain tumors can lead to a better development of therapeutic and prognostic approaches. The second objective is to determine how a detailed study of immunological parameters in human brain tumors can be used to develop diagnostic and immunotherapeutic modalities for these patients. The third objective is to develop a program for rational planning of chemotherapeutic usage based on individual patient response.

## II. MATERIALS AND METHODS:

A. Tissue culture technique: The primary method used for this project is that of the tissue culture of human brain tumor cells. In this technique cells removed at operation are placed in a medium consisting of F10 with 10-12% fetal calf serum. The tissue removed at surgery is minced to 1 mm chunks and then explanted into plastic Falcon bottles with appropriate amounts of the standard medium. After cellular outgrowth begins the medium is changed regularly and then cells are subcultured when needed with .25% trypsin. The cells are passaged and harvested to provide the basic cellular material for all of our other techniques.

A new primate glioma model developed in the laboratory of Dr. John Sever (utilizing JC virus from human prognosis multifocal leukoencephalopathy) is now being used to extend the human studies.

B. Biological techniques: The detailed characterization of the tissue culture cells requires performance of PPLO or mycoplasma testing to determine that the cells are free of contamination. It also requires light and electron microscopy, biophysical and biochemical studies. The electron microscopic studies involve scanning, transmission, surface replica and x-ray spectrometry studies to evaluate the surface and intracellular characteristics of the tumor cells. Direct correlations can thus be made of surface characteristics with malignancy. A morphometric comparison of the number and type of junctional complexes formed between cells of several cultured lines has been carried out. An automated image analysis system based on a Bausch and Lomb FAS II basic processing unit has been developed and put into use to provide quantitative measures of cellular and subcellular morphology as well as to surface mapping in various states including before and after immune and chemotherapeutic attack. Increasing effort has also been devoted to finding new methods



to study the extracellular mucopolysaccharide/glycoprotein coat of glioma-derived cell lines. Successful utilization of aqueous soluble plastic or embedding compounds has been a significant accomplishment. Pyro-antimonate staining of intracellular calcium deposits for x-ray spectroscopy of intracellular  $\text{Ca}^{++}$  in tumor cells is being utilized as well. Biophysical studies include microelectrode recordings with single cell determination of cell brain resting potential, time constant and cellular resistivity. The biochemical studies including analysis of S-100, glial fibrillary acidic protein, myelin basic protein gangliosides collagen synthesized and released proteins and glycoproteins, and flow cytofluorometric separation of tumor DNA are performed on cultures at varying time periods from initial explantation. Characterization of the degree of functional malignancy can be accomplished by means of using the cell's ability to grow in low serum (.5%), ability to grow in soft agar and to penetrate nucleopore filters. The cells may also be studied in an animal model system such as the immunosuppressed hamster to determine whether they are able to produce a tumor similar to that seen in the patient.

Dr. Eugene Quindlen and Dr. Yoshio Moriya, a Visiting Fellow with the SNB, have focused their efforts on biochemical and immunological techniques useful in the detection of glial markers in tissue culture cells. Both S-100 protein and glial fibrillary acidic protein have been purified and appropriate antisera raised to proteins. Using isoelectric focusing and crossed immunoelectrophoresis, GFA in astrocytoma cells has been separated, identified and quantitated in tissue culture cells.

Rockett immunoelectrophoresis has not been as useful in identifying low concentrations of S-100 in cells. Therefore, an enzyme-linked immunoassay is under development for the detection of S-100 as well as other putative markers present in glial cells. However, the antisera developed to these proteins have already proved useful in establishing clinical and pathological diagnoses in fresh sections of tumors using the immunofluorescent technique.

For GFA and FN studies, the immunofluorescence procedure for GFAP, developed by Dr. Paul McKeever in the SNB has been modified to be used alone for fibronectin (FN) and a staining of FN and GFAP in the same preparation with short preparation time (15-20 min) has been worked out to be used for diagnostic purposes. The GFAP-peroxidase-anti-peroxidase (PAP) protocol of Mr. Steve Laverson has been modified to give a result for GFA in 2 hours and for FN in 25 min. This method provides permanent sections. These two methods work on cultures, cryostat sections of OP-material, autopsy (up to 14 hours postmortem has been tried) and frozen-stored material. For intracellular localization of FN the method of Yamede et al has been adopted in a simplified version.

C. Immunological studies: Two basic types of humoral immunology studies have been carried out. The first involves a microcytotoxicity assay. In this microcytotoxicity assay cells are transferred from Falcon plastic flasks (in a suspension of approximately 30-50 thousand cells per cc) to

the individual wells of a Falcon microtiter plate using a Terasaki syringe at an approximate 100 density of cells per well.

These cells are allowed to establish themselves for 12-18 hours and then immunological testing with antibody and complement is carried out. The antibody can be either whole prepared from serum or serum which has been fractionated into its globulin components. The complement used is a combination of human pooled serum or human cord serum with rabbit serum as a primary complement source. It is important that the rabbit serum be obtained from rabbits approximately 4 to 6 weeks of age. The complement preparation is added approximately 1 hour after the addition of the serum and the plates are then incubated for 18 hours at 37°. Finally, the plates are stained with hematoxylin-Giemsa and the cells counted. By careful arrangement of the cells in the plate, it is possible to analyze the effects of 4 to 6 individual patient sera on a line simultaneously. This approach allows for excellent statistical quantitation and determination of what is known as the cytotoxic index (C.I.). This index is essentially the ratio of cells that have been eliminated by the immunological interaction to those in the untreated control wells. A cytotoxic index of .2 or above is generally statistically significant. Precise statistical determinations are made on each set of observations. The counting of cells in the plates has now been fully automated using an image analysis computer and scanner which also does the statistical analyses.

Immune adherence testing is carried out with the use of antibody from specific patients, target cells and red blood cells. The antibody-coated red blood cells attach to the surface of a target cell and when one sees adherence of numbers of red cells to a given target cell it is considered to be a positive response. This technique has the advantage of allowing careful serial titer dilution and also permits absorption with various cellular or tissue components.

Cultured human glial tumor cell lines of various grades (II-IV) have been treated with differentiating agents, either dibutyryl adenosine cyclic monophosphate (dbCAMP) or dimethylformamide (PMF). The cell lines have been analyzed for morphologic changes, and growth curves have been plotted.

In planning a rational program of immunotherapy against brain tumors, knowledge concerning the ability of brain tumors to elicit cell-mediated immune responses and concerning the susceptibility of brain tumors to be destroyed by various cell-mediated immune mechanisms is critical. However, very little is known regarding these questions. The initial goals in this work have been (1) to develop assays for monitoring the cellular immune status of brain tumor patients prior to and during immunotherapy and (2) to develop *in vitro* model systems for studying the ability of brain tumors to elicit and be destroyed by cell-mediated immune mechanisms, including lysis of tumor cells by specifically-immune cytolytic T lymphocytes (CTL), by natural killer cells (NK cells), by activated macrophages, and by antibody-dependent killer lymphocytes

(K cells). Our initial work has focused on the potential role of specific cytolytic T lymphocytes in cell-mediated immunity to brain tumors. The possible importance of other cellular immune effector mechanisms will be examined in the future. Dr. Maurice Gately, who is directing this work, has previously had extensive experience in studying the generation and mechanisms of action of cytolytic T lymphocytes and in studying lymphokines, the soluble mediators of cellular immunity. Thus the present work represents a direct extension of his prior interests.

The initial requirement in these studies was to develop a reliable and quantitative assay for measuring cell-mediated immune lysis of human glioma cells. The  $^{51}\text{Cr}$  well, and the rate of spontaneous release of  $^{51}\text{Cr}$  from all glioma cell lines studied thus far has varied from 20 to 30% of the total releasable  $^{51}\text{Cr}$  in 24 hours. Thus, it is readily possible to use  $^{51}\text{Cr}$  release to assess the ability of lymphocytes to lyse glioma cells over a 24 hours period.

Lymphocytes are isolated from human peripheral blood by centrifugation of Ficoll-Hypaque. This is followed by further centrifugation on sucrose gradients to remove contaminating platelets. Cytotoxic lymphocytes are generated by incubation of responder and stimulator lymphocytes and glioma cells in 2 ml volumes of culture medium containing 5% human AB serum in the wells of Linbro or Costar tissue culture plates. Stimulator lymphocytes receive 2000 R of gamma irradiation prior to culture and glioma cells receive 10,000 R of gamma irradiation. Responder lymphocytes are cultured at a density of  $1-2 \times 10^6$  cells/well. Optimal glioma cell density has been found to be  $0.5-1 \times 10^5$  cells/well for the cell lines studied thus far. Irradiated stimulator lymphocytes are most often used at a density of  $1 \times 10^6$  cells/well. Cytotoxic cells are harvested after 7 days of culture, and their ability to lyse  $^{51}\text{Cr}$ -labeled glioma targets is measured.

Glioma cells to be used as targets in lytic assays are incubated with 100  $\mu\text{Ci}$   $^{51}\text{Cr}$  in 1 ml of culture medium for 1 hour at  $37^\circ$ . The cells are then washed extensively to remove any  $^{51}\text{Cr}$  which was not incorporated into the cells. One tenth ml of cell suspension containing  $10^5$  glioma cells/ml is seeded into each well of a Falcon Microtest II culture plate. The cells are allowed to establish themselves for 18-24 hours. The culture medium is then aspirated from each well and replaced with 0.2 ml of lymphocyte suspension. Lymphocytes are incubated with glioma cells for 24 hours at  $37^\circ$ . At the end of this time, 0.1 ml of culture medium is withdrawn from each well, and the amount of  $^{51}\text{Cr}$  contained in each sample is measured in a gamma counter. The total amount of  $^{51}\text{Cr}$  which was contained in the glioma cells incubated with lymphocytes is calculated as  $(e - c)/100 - c$  where  $e$  is the percentage of  $^{51}\text{Cr}$  released from glioma cells incubated with lymphocytes and  $c$  is the percentage of  $^{51}\text{Cr}$  released spontaneously from glioma cells incubated alone.



D. Chemotherapy studies: The chemotherapeutic studies are carried out by means of a similar microtiter system in which various chemotherapeutic agents can be studied, primarily the nitrosoureas BCNU, PCNU, and cis-platinum and methotrexate. The target cells from individual patients are exposed for varying periods of time to these therapeutic agents. Direct observations can be made of cell killing by means of phase microscopy and time-lapse microphotography as well as by cell counting similar to that done in the immunological techniques. A cytotoxic index can be established for each agent at each concentration. The cell killing at concentrations closest to those achievable in patients can then be compared to actual clinical responses seen in patients who are receiving such therapy. The automated counting system developed for this technique has been written up and submitted for publication.

A new in vitro chemotherapy technique enabling the testing of agents insoluble in aqueous media has been developed. Utilizing the aqueous insoluble drugs in the solid phase and the cell membrane itself as the solvent, this assay has made it possible to test a much wider range of potential antiglioma agents than was previously possible.

Drugs tested over this year have included AZQ, rapamycin, cis-platinum, BCNU, spirohydanotin, phenytoin, WR-2721, PCNU and CCNU. For the solid phase testing each drug is prepared freshly in ethanol for each test at an initial concentration of 1000 mg/ml. Subsequent dilutions are also prepared with ethanol to provide concentrations of 100 mg/ml, 50 mg/ml, 25 mg/ml, 10 mg/ml, and 0.1 mg/ml. Ten of each of these dilutions are then placed in a series of 6 microtiter wells, according to the same scheme as for the aqueous phase testing (see Kornblith and Szytko, 1978). Control wells receive 10  $\mu$ l of ethanol only. All ethanol is then evaporated rapidly (within 5 minutes) from the plates under a laminar flow hood, thereby depositing the agent to be tested on the bottom of the well. Total drug deposited for each of the drugs listed above is thus 1  $\mu$ g, 0.5  $\mu$ g, 0.25 mg, 0.10 mg, 0.01 mg, and 0.001 mg per well surface (.75 mm<sup>2</sup>). From examination of the drug deposition at the well base, the bulk of the drug (including stain deposition and patterns of cell killing on the well base surface) is deposited over a central base area of approximately .65mm<sup>2</sup>.

After the drugs have been precipitated on the well base surface, the cells prepared as described above are added to the wells to provide 70 cells/well. Thereafter the plates are covered and placed in a 95% Air, 5%CO<sub>2</sub> atmosphere at 37° and for periods of 24, 72, or 168 hours. At the end of the test period the plates are removed from the incubator, fixed in methanol and stained with Giemsa. A cytotoxic index,  $1 - \frac{\# \text{ cells treated wells}}{\# \text{ cells control wells}}$  is calculated from the counts of living

cells in each of the wells utilizing techniques previously described. Statistical significance is ascertained by the Student t-test. Counts for all cells were done either by an observer or by a Bausch and Lomb FAS-II Omnicon Image Analysis System detecting nuclei in stained cell populations.

Loss of a particular drug by degradation during its solubilization and evaporation of the solvent was of concern, especially for a drug such as BCNU, whose half-life under a variety of conditions is known to be of the order of minutes. Accordingly, intervals from drug solubilization to cell plating of 7, 20, and 90 minutes were tested to evaluate possible loss of each drug with response at the shortest time interval acting as the effective drug control.

E. Biological growth control and/or "differentiation": A potential important variation of the chemotherapy assay is the use of biological growth modifiers such as cyclic AMP, dimethylformamide and interferon (both glioma cell produced and human fibroblast types). Other modifiers with direct effects on response to other agents are also being studied. These include sulfhydryl-containing reagents and calcium ionophases (A23187). All agents can be tested in the microtiter assays described above either as acute treatment, chronic treatment, or modifying agents for another drug. For human fibroblast interferon tests utilized 1-10,000 units/ml. To induce interferon production a poly ICLC cycloheximide, actinomycin D stimulus together with human IF primer was utilized to induce IF production by the glioma derived cell lines. IF assays were conducted in collaboration with Dr. Christian Anfinsen's laboratory according to standard protocols.

F. Neuropathology: Correct histologic diagnosis and grading of human brain tumors is necessary to determine proper therapy. With the cooperation and histologic support of Drs. Jose Costa, Alan Rabson and Marious Valsamis of the NCI, Dr. Paul McKeever continues a diagnostic service for the Clinical Center. Clinical neuropathology has been improved at the NIH during the period of this Annual Report. At present, three neurosurgery residents and five pathology residents are receiving neuropathology instruction. Plans to formalize a neuropathology program are underway. Classification of astrocytomas by grade according to the criteria of Kernohan and Sayre is accomplished on all patients' tumors including those admitted for radiotherapy or for immunotherapy and on consultation cases. Consultations have increased from zero to about six per month. An effective working relationship has been established with the laboratory of Pathology at the Clinical Center and with the Neuropathology Division of the Armed Forces Institute of Pathology, extending histologic and diagnostic capabilities and increasing the variety of material available for analysis.

Frozen section diagnosis has been improved with the use of immunocytochemistry to localize tumor antigens, specifically glial fibrillary antigen and fibronectin. Recognition of glial fibrillary antigen in neoplastic cells allows immediate differentiation between fibrillary gliomas and schwannomas or meningiomas. Staining for fibronectin offers similar power of discrimination by localizing meningotheial cells, Schwann cells and fibroblasts.



## III. MAJOR FINDINGS:

Major findings over the past year can be divided into 1) cell biology/characterization; 2) chemotherapy; and 3) immunology categories.

Ultrastructural studies of CNS neoplasms encountered on the clinical service of the SNB have defined a new constituent of at least one ganglioglioma, bihelical filaments. Combined light, electron and scanning electron microscopy of epithelial cysts in the central nervous system have contributed evidence for a non-epithelial origin of so-called supracellular epithelial cysts. In fact these solitary cysts, lined almost entirely by squamous epithelium may be derived from Rathke's pouch similar to craniopharyngiomas.

Rare CNS tumors have provided new information on the surface topography of their predominant cell types. Of particular interest are the scanning microscopic appearance of the plasmalemma over lipid inclusions in the capillary hemangioblastoma and of membrane organelles in the choroid plexus papilloma.

Characterization of human lines cultured from gliomas has utilized electron microscopic, biochemical and immunologic methods. Rare individual differences have been seen between small percentages of cells from certain gliomas and fibroblast controls. These include desmosomes and single cilia.

Different patterns of surface mucopolysaccharide staining of human glioma-derived cells in culture have also been observed. One cell population is characterized by a thick ( $> 1$  cell diameter) halo which keeps other cellular elements (such as lymphocytes) at a distance. This halo contains ruthenium red (+) and Alcian blue (+) material on fine and extremely long microvilli. This extracellular matrix, which may have therapeutic implications, especially for immunotherapy, requires further study. Active pinocytosis has also been observed at the surface of these glioma derived cell lines.

Patterns of protein synthesized and released by cells cultured from human gliomas have been described. These patterns were compared with the patterns of syngeneic fibroblasts. The patterns of paired human lines studied thus far have had many similarities. In an effort to determine whether the similarity of released proteins from human gliomas and syngeneic fibroblasts reflects a general identity of these two cell types, the released proteins of prototype animal lines were also compared. The C6 rat glioma, an established clonal glial cell line, was compared with syngeneic fibroblasts and with rat fibrosarcomas. Differences are apparent between C6 and these other fibroblastic lines. Some of these samples have been compared on gel electrophoresis and protein bands at the same migration distance and same molecular weights have been found in the surface and in the medium in both human cell lines and in

rat glioma. This indicates that some proteins found in the medium of cultured glioma cells may be shed from the surface of these cells.

On the basis of the described experiments and of published data, it was determined that more definitive probes of glial differentiation were needed. Of equal importance reliable probes for fibroblasts were essential so that these cells could be identified adequately. Immunocytochemistry and immunofluorescence were chosen for their ability to distinguish cells on an individual basis. With the help of Mr. Steve Laverson and Dr. Bibie Chronwall, immunoperoxidase, fluorescein and rhodamine stains for glial fibrillary acidic protein (GFAP) and for fibronectin (FN) were accomplished and applied to gliomas in tissue, in early explants and in culture at various passages. Results on astrocytomas in tissue as more fully elaborated by Dr. Chronwall, show that astrocytoma cells are GFAP + and FN - while endothelial cells and fibroblasts are GFAP - and FN +. Significant points are: Anti-FN will distinguish non-glial from glial neoplasms in 8 min. The combined use of anti-FN and anti-GFAP will give a more extensive answer in 20 min. FN (+) cells are perivascular and GFA (+) cells are parenchymal in a malignant astrocytoma. In a meningioma every cell is surrounded by FN.

The results from tumor material could explain some of the observed heterogeneity of "glioma" cell populations in culture - the cells come from different parent cells. Of the seven astrocytoma-glioblastoma lines investigated, one (Richardson) clearly has three populations of cells (FN+ 60%, GFAP+ 40% and a few non-stained cells), the others are FN + but GFAP- and with some non-stained cells. The tumor of Drotleff is GFAP+ in the parenchyma and FN+ in the vessel walls. One explant bottle showed GFAP+ cells in the center of the explant-overgrown with FN+ cells which also migrated out from the explant. Among the cells in the periphery one GFAP+ (and FN-) cell was found. The FN/GFAP method could be useful when setting up tumor cultures and to follow their development and changes with time--a major goal of this program. Work is now underway to provide additional culture conditions which favor retention of GFAP (+) astrocytoma cells in culture and to clone for such astrocytoma cells.

Correlated analysis of microfluorometric (MFM) DNA content and karyotyping has proved to provide more accurate cell kinetic parameters of cultured human clonal glioma derived lines. The present limitations of MFM studies may be due to difficulty in separating the cells in G2M phases (post DNA-synthetic phase and mitotic phase) of a diploid cycle from G1-cells (pre-DNA synthetic phase) of a tetraploid cell cycle. Cytogenetic analysis, however, solves this limitation by a precise karyotyping and chromosomal distribution in the cell population. By the same token, cytogenetic studies also seem to have limitations--the possible existence of non-proliferating cells and aneuploid cells which are lost from analysis. Thus the MFM and cytogenetic studies are complementary to the other. The cell lines so far examined (combined study 10 lines; MFM study, 16 lines) have been categorized in three

groups, namely near-diploid, aneuploid and near-tetraploid. All cell lines showed a DNA-histogram composed of two major peaks (G1 and G2M phase), which implied that every cell line can be estimated as a single population in MFM analysis. The histograms of DNA-content are obtained by mithramycin-ethidium-bromide DNA-specific double staining and co-running chicken erythrocytes as an internal standard. Co-running sample was crucial for measurement of relative DNA content for comparison with the position of the G1 peak. This system provides the ratio of DNA content of sample to standard known DNA content (the ratio of human lymphocyte DNA to standard is  $2.7 \pm 0.2$ ).

Tumor cell lines indicate this ratio to be between 3.0-6.0. Cytogenetic studies reveal a wide range of chromosomal aberration (missing chromosome, P, Q deletion, trisomy, tetrasomy, and marker chromosome) and ploidy. The comparative relative DNA content is approximately consistent with the chromosomal number histogram and the relative computed value from each chromosomal DNA (in A,B,C,D,E,F,G,X,Y). A slight discrepancy between DNA ratio and exact number of chromosomes occurred in high chromosomal numbered lines (near-tetraploid).

Findings in the positron emission computed tomographic scanning of patients with gliomas have revealed a correlation of pathological grade of the tumor with glucose metabolism. We have studied this phenomenon in tissue culture as well to ascertain the dynamics of glioma cell glucose metabolism. Findings thus far include:

- (1) The rates of [ $^{18}$ F]-2-deoxy-d-glucose uptake measured in tumors in situ (by PECT scanning) is comparable to the rate of glucose uptake in tissue culture lines derived from tumors at the time of surgery. Differences in the rates of glucose uptake between lines derived from different histological grades of tumor were not observed.
- (2) Fifty percent or more of the glucose consumed appears as medium lactate or pyruvate. This suggests that no more than 50% of consumed glucose is aerobically metabolized. The aerobic glycolytic rate does not correlate with the histological grade of the tumor of origin.
- (3) Glutamate and glutamine (which together comprise approximately 1.6 mM in medium) are taken up with a rate comparable to the rate of glucose uptake; this may constitute a significant carbon source for oxidative metabolism.
- (4) Each cell line has a characteristic glycogen set-point level. The levels appear to be stable, and not significantly affected by the administration of fresh glucose (refeeding).
- (5) We have characterized the cell surface receptor systems in selected astrocytoma grades III and IV, by administering

pharmacological agents and scoring for glycogenolysis and/or elevations in cyclic AMP. Each cell line has a characteristic pattern of agents which elevate cyclic AMP or cause glycogenolysis. Most lines show a glycogenolytic response and elevation of cyclic AMP in response to catecholamines, and glycogenolysis in the absence of changes in cyclic AMP after treatment with the calcium ionophore A23187.

Glucose uptake by both neuron and glia in the central nervous system may involve modulation by a direct coupling of the neurotransmitter-receptor system to the glucose transport system. To study this possibility in glial cells, the rat C6 glioma cells are known to have beta-adrenergic receptors which are coupled to adenylate cyclase.

When C6 glioma cells were stimulated with  $10^{-6}$ M isoproterenol, uptake of  $^3\text{H}$ -deoxyglucose decreased by 50% in the first 30 min. (19.75 fmoles/min/mg protein, SEM  $\pm$  2.11, N=4,  $P < 0.02$ ) and then increased by 200% up to 150 min. (48.09 fmoles/min/mg protein, SEM  $\pm$  1.47 N=4,  $P < 0.0001$ ) in comparison with unstimulated control 30.98 fmoles/min/mg protein).

This increased glucose uptake appears to be mediated through beta-adrenergic receptors, since the order of potency of various catecholamines is isoproterenol > epinephrine > norepinephrine. In addition, the d-isomer of isoproterenol was much less active than the l-isomer and the beta-adrenergic agonist, propranolol, but not alpha-adrenergic agonist, phenoxybenzamine, inhibited the increase in glucose uptake.

C6 glioma cells respond to the continuous treatment (2-150 min.) of l-isoproterenol ( $10^{-6}$  MOL) with a rapid increase of intracellular cAMP level (2478 pmoles/mg protein) from basal level below 36 pmoles plateau (30-40 min) and then rapid fall (110 pmoles/mg protein 150 min). The decrease of intracellular cAMP in the late phase strongly suggests uncoupling of adenylate cyclase in the system, and thus the accelerated uptake of deoxyglucose might be related to the biochemical change in the membrane which cause the desensitization in the system.

#### Chemotherapy

In the aqueous microtiter assay 21 new glioma lines have been tested for sensitivity to BCNU. The data derived to date for these lines continue to confirm the fact previously determined that 60% of the lines are sensitive to BCNU. PCNU has been further tested but without any significant cytotoxicity. Cis-platinum has been tested in 10 lines and all are highly sensitive to this agent, although a 72 hour observation period is required to see killing. Methotrexate is also being tested in the aqueous assay.

In the solid phase assay our first studies involved five drugs on five glioma-derived cell lines to determine the suitability of the method and the relative effectiveness of each of these drugs. The drugs tested in these initial studies were BCNU, PCNU, spirohydantoin, rapamycin and aziridinbenzoquinone (AZQ). Findings have been:



- (1) The surface assay is a practical and reliable method for the in vitro testing of aqueous insoluble chemotherapy agents;
- (2) Comparison of the aqueous and solid phase test for BCNU, which can be used in both, reveal comparability;
- (3) Aziridinybenzoquinone(AZQ) appears to be an effective anti-glioma chemotherapy agent which to date has shown significant cytotoxicity against all glioma lines but not all fibroblast control lines. It, in some cases, shows increased effectiveness with time of exposure such that by 168 hours doses not effective at 24 hours may be quite toxic (cytotoxic index  $\geq 0.5$ ).
- (4) Spirohydantoin has shown cytotoxicity when applied to glioma cells, but does so only after a minimum of 24 hours with full antitumor toxicity only apparent at 168 hours after initial exposure. This is comparable to the findings we have previously reported for phenytoin.
- (5) Rapamycin, a long carbon-chain antibiotic, has shown some activity against glioma-derived cell lines in this tissue culture assay. It appears to be less effective than AZQ in all but one.
- (6) Drug decay is an important consideration in both the solid and aqueous phase techniques. This is especially true in the case of BCNU where delay in application of the drug to the cells of the order of 15 minutes results in a loss of activity. Formal half-life studies are being undertaken.
- (7) PCNU has not yet proved effective in the solid phase test.

More extensive studies of a series of 20 glioma-derived cell lines with respect to their sensitivity to AZQ reveals sensitivity of all lines to AZQ although there are clear differences from line to line. For control fibroblast lines, examples of both sensitivity and resistance exist. Preliminary studies to determine the nature of AZQ's cytotoxic action have shown both nuclear and mitochondrial changes. It has been postulated, although not established, that AZQ is an alkylating agent. Its quinone structure also suggests a mitochondrial site of action and the mitochondrial changes seen in transmissions electron microscopy in AZQ-treated cells appear to support such a notion.

Phase I and early phase II clinical trials have been initiated with AZQ in collaboration with the NCI. Ten patients are now receiving AZQ on Day 1 and Day 8 of a monthly cycle. Some of these patients have shown temporary improvement in their clinical status over periods of up to several months, but the determination of the utility of AZQ will require substantially more patients and a randomized study now being organized.



Several other new, potential antiglioma agents have undergone at least preliminary testing using either the aqueous or solid phase micro-cytotoxicity assay. Of these, cis-platinum, which has now been tested in 6 lines, shows dramatic toxicity in vitro at very low dose levels. Further studies to confirm this finding are in process.

Factors determining a cell's response or non-response to the nitrosourea (BCNU) have also been the subject of continued study. The findings with DNA methylation repair studies have not changed dramatically from last year's report. Only one of SNB's glioma lines tested has been DNA repair deficient and, overall, including outside lines tested by Dr. Rufus Day of the NCI, at most half of the 13 glioma lines tested to date have a repair deficiency.

Pursuing the notion that cytoplasmic factors, in addition to nuclear factors, may be critical to sensitivity to BCNU, we have compared BCNU-sensitive and insensitive glioma lines in their responses to BCNU by microcinematography, microvideography, and scanning and transmission electron microscopy. In the two lines studied to date the BCNU-sensitive line has shown marked surface membrane blebbing in response to BCNU, whereas the insensitive or resistant line shows no such pattern. This material, being submitted now for publication, is consistent with the hypothesis that non-nuclear factors are important to the BCNU response. Pursuing this with  $^{14}\text{C}$ -BCNU we have determined that there are different patterns of intracellular protein and DNA binding between the two lines as well.

The sensitivity to BCNU of a given tumor cell line is likely to be of multifactorial origin. Factors include membrane properties, cytoplasmic binding sites, cytoplasmic inactivation or metabolism capability for DNA repair, and so forth. A specific mechanism falling under the cytoplasmic factor category is that of the role of sulfhydryl moieties in nitrosourea effectiveness. Previously published data have indicated that increased sulfhydryl (SH) moiety content in the cytoplasm increased the effectiveness of BCNU. We have initiated a series of experiments to alter intracellular (SH) content utilizing glutathione, mercaptoethanol and cysteine. Decreased cytotoxicity compared to controls has been found in this experimental series and suggests increased inactivation of the parent compound or its metabolites. This, in turn, suggests that (SH) content may have a role in determining sensitivity or resistance. However, this hypothesis must be tested further as other explanations could be said to be equally valid. The major concept is that we now have the beginnings of a detailed assessment of the cellular basis of sensitivity or resistance to chemotherapy. This in turn should eventually lead to clinical applications either in the areas of chemotherapy modifiers or multiagent protocols.

With respect to chemotherapy modifiers, one other area has also been examined -- that of the interaction of radiosensitizers with the nitrosoureas. BCNU has been tested in the microtiter assay in

combination with misonidazole. Testing was done in the solid phase assay as well as the aqueous phase assay. Control plate cells (treated with BCNU alone) were compared with cells treated with BCNU and misonidazole ( $10^{-3}M$ - $10^{-4}M$ ). To date there has been no difference in the C.I.'s of the control and treated plates.

Finally, with respect to in vitro chemotherapy assays, the question remains as to the correlation of the in vitro measures of cell killing with clinical response. In other words, are the in vitro assays predictive of clinical response or non-response? Published in Cancer this year (Cancer 47 : 255-265 ) was the initial series of 14 patients for whom such a correlation was attempted. In this study non-response or resistance was predicted in all the cases (5/5), whereas sensitivity in vitro was predictive of clinical response in 6/9 cases. These data, although, of course, far too limited for definitive correlation are encouraging enough to support further correlative studies on larger series of patients and this is, in fact, in progress now as a continuing SNB project.

#### Immunology

In the humoral immunology assays SNB studies have continued to indicate high specificity for autologous patient glioma cytotoxicity testing. Not only is there a highly specific patient immunological response to gliomas, but, very importantly, the incidence of positive response is highest in patients with lower grade (Grade I-II astrocytomas), rather than the more malignant Grade III-IV tumors, suggesting that the more malignant tumors escape immune detection and destruction. A further correlation is that a positive humoral antibody microcytotoxicity response can be correlated with longer survival times. The immune adherence assay, however, does not correlate with clinical survival. Finally, the immune adherence and microcytotoxicity assays have been determined to depend on different immunoglobulin classes, so that the mechanisms of tumor-immune defense and lysis can be approached more effectively.

In the current series of 42 patients with glial tumors, 44 percent had detectable cytotoxic antibody to their own tumor cultures. In 15 cases, autologous fibroblasts were cultured and these were consistently negative. Analyzing results in terms of histology, 75 percent of astrocytoma cases were seropositive compared with 17 percent positives in patients harboring the more anaplastic glioblastoma multiforme.

When the serum from these patients was tested on an allogeneic common target glioma line, there was not a statistical difference in activity between the astrocytoma and glioblastoma sera, as had been seen autologously.

When this population is subdivided by tumor grade, the results become more interesting. Seventy percent of patients with an astrocytoma

grade I-III elicit a positive response whereas only 23% of patients with a grade IV glioblastoma seem to have autologous antibody. The lower grade tumors are comprised of younger people with an average age of 38, while the average age of the glioblastoma group is 57. Not only is there a direct correlation of tumor grade, presence of antibody and age, but an additional correlation with survival exists. Those patients who elicit a positive humoral response have an average survival of 23.6 weeks as opposed to the group without this response whose survival averages 10.8 months. The obvious question which exists is whether it is possible to alter tumor cells and ultimately affect a patients' immune reactivity. Since the lower grade tumors are more often positive, it would be interesting if the more malignant tumors could be differentiated. It has been possible to obtain fibroblast in 16 cases and all have been negative when tested with autologous serum.

The serological results were then considered in terms of survival and curves were constructed which showed that the presence of cytotoxic antibody correlated with survival in a highly significant manner ( $p=0.0007$  Gehan-Generalized Wilcoxon), this, of course, reflecting the relationship of seropositivity with tumor grade. Age at diagnosis also correlated closely with outcome and with serum cytotoxicity ( $p=0.00015$ ).

The highest incidence of positive responses occurred in pre- or intra-operative sera. At this time all cases were receiving corticosteroids. In most patients subsequent sera become negative, coinciding with an administration of radiotherapy and chemotherapy (CCNU).

A positive anti-glioma serum was absorbed with the corresponding autologous fibroblast culture and when retested on the tumor cells retained its cytotoxic activity.

Cytotoxic antibody activity appears to be related *pari passu* with the degree of glioma neoplasia and subsequent clinical course. In accordance with current concepts, we suggest that our finding indicates altered, host immunocompetence, a phenomenon now widely described in patients harboring various tumors including gliomas.

The previously reported decrease in immune responsiveness of glioma patients to recall skin testing and lymphocyte transformation to P.H.A. indicates a general suppression of non-specific antigenic stimulation. We would emphasize that allogeneic cytotoxic activity was unimpaired in the patients with absent response to their own tumors. This suggests the absence of idiotypic antibody in these individuals, who nevertheless retain a response to a cell line which displays a range of common neuroectodermal antigens.

This finding also excludes failure of general immunocompetence related to age. Others have tested the concept of age-related energy in cancer patients and find no relationship.

The considerable evidence that circulating immune complexes play a role in abrogating anti-tumor immunity and adversely affecting prognosis suggests that antibody sequestration in complexes or tumor-bond form may be an explanation of the present findings. Indeed, it has been recently reported that IgG binding activity is significantly elevated in high compared to low grade glioma patients and that this is associated with a decreased survival.

The antigen(s) recognized by this cytotoxic autoantibody appear to be restricted to the cultured glioma cells since 15 paired fibroblast cultures are negative on direct testing and absorption of serum failed to alter reactivity when retested on the autologous glioma. These findings are consistent with a recent study in which restricted, common and oncofetal glioma surface antigens were demonstrated by absorption studies using various binding or complement fixing assays and the presence of H.M. antigen was excluded.

Other investigative work in the area of humoral immunology examined membrane surface characteristics of human glioma target cells in the process of humorally mediated complement dependent cytolysis. Three specific membrane surface characteristics were studied: (1) alteration of target cell antigenicity after trypsinization; (2) cytotoxicity of serum alone; (3) cell morphology after interaction with serum and complement using the electron microscope. Two glioma tissue culture lines were treated with sera from six patients plus normal pooled human sera. The microcytotoxicity assay was used to examine antigenicity, varying incubation periods at twenty-four hour intervals. The cytotoxic index was calculated for each serum used. The cytotoxic index was found to be greater when plates were incubated more than forty-two hours, using the cytotoxic serum on both target cell lines. The same serum alone also produced marked cell lysis after the forty-two hour incubation period. Scanning electron microscope preparations were made with the two glioma lines and four of the above sera. Cell diameter shrinkage and membrane edge retraction were most evident after addition of serum alone or serum plus complement. The next most common characteristics included formation of blebs and pseudopodia. All of the above surface membrane characteristics could be found using cytotoxic and non-cytotoxic sera. Albeit, the cytotoxic sera produced the greatest amount of cell shrinkage, in addition to shrunken cells lacking any appendages whatsoever. Normal pooled human serum produced only cell shrinkage consistently.

Another area of exploration has been the possibility of producing changes in antigenic expression and/or lytic susceptibility via alteration of the state of differentiation of the tumor cells. Dibutyryl cyclic adenosine monophosphate (dbCAMP) and dimethylformamide have been used in these studies. With dbCAMP, cell processes became more prominent and phase contrast microscopy showed definite cytoplasmic skeletal structure. DMF generally causes cell elongation. Growth curves are depressed with each of the differentiating agents.



To study the possibility of differentiating agents' effects on humoral immunity, the treated and untreated cells as well as parallel control lines have been subjected to microcytotoxicity assay and immune adherence study. Enhanced cytotoxic indices and increased immune adherence with each of the differentiating agents has been shown for some lines and suggests the value of further studies in this area.

The finding that glioma cells release proteins into the extracellular medium has been an important advance as well because of the chance it offers for study of glioma cell antigens. In one model system rat C6 gliomas and human cultured lines (8 line) were labelled with  $^3\text{H}$ -fucose for biosynthesis of glycoproteins. These biosynthesized glycoprotein were mainly incorporated into the membrane.

Forty to 56% of these membranous glycoproteins proved to be released into medium by turnover of membrane macromolecules, and SDS-PAGE reveals glial released glycoproteins (GRGP), and cellular glycoproteins as numerous bands.

We have tried to immunoprecipitate cellular-retained glycoprotein and GRGP's with rabbit-anti-C6, and human autologous serum C6; rabbit-anti-C6 serum, sheep-anti rabbit IgG, human lines; autologous serum, rabbit-anti-IgG). Major glycoprotein immunoprecipitated from C6-cell-retained glycoprotein was a 110,000 dalton glycoprotein, and the released immunoprecipitable glycoprotein peak was a 125,000 dalton glycoprotein. For the human gliomas tested for retained glycoprotein, only one line has immunoprecipitable glycoprotein (95,000 dalton). The released fraction did not have any immunoprecipitable macromolecules.

Work in cellular immunology has continued to focus on two areas: (1) monitoring of the cellular immune status of glioma patients during the course of their disease, and (2) examination of the ability of human glioma cells to elicit and be destroyed by cytotoxic lymphocytes in vitro.

Freshly isolated peripheral blood lymphocytes from each of 6 glioma patients failed to lyse autologous glioma cells to an extent significantly greater than normal control lymphocytes when assayed as described above at lymphocytes:glioma ratios as high as 100/1. Likewise, lymphocytes from 6 or 7 glioma patients also failed to lyse allogeneic gliomas. Peripheral blood lymphocytes from one glioma patient were found to cause substantial lysis of 3 of 3 allogeneic glioma lines and 1 of 2 allogeneic fibroblast lines but did not lyse an allogeneic colonic adenocarcinoma or an allogeneic myeloid leukemia to a greater extent than lymphocytes from normal controls. Neither autologous glioma nor autologous fibroblasts were lysed. The lytic activity against allogeneic gliomas was shown to be mediated by T lymphocytes, not by natural killer cells. It has been maintained at essentially constant levels throughout the 10 months during which this patient has been followed



immunologically. The nature of the antigen recognized by this patient's lymphocytes and whether or not this reactivity is related to the patient's disease are at present unknown. However, similar reactivity has not been observed using lymphocytes from any of the 32 normal donors which have been tested thus far.

All patients who have been studied early in the course of their disease have shown little or no depression of lymphocyte function as measured by the ability of their lymphocytes to respond to the mitogens PHA, Con A, and pokeweed mitogen or to allogeneic lymphocytes in mixed lymphocyte cultures. However, two patients who were followed to the time of their death showed declining, and in one case virtually absent, lymphocyte function as their disease progressed. Three patients who received immunotherapy consisting of injections of irradiated cultured autologous glioma cells failed to show any increased cytolytic lymphocyte activity against the autologous tumor, or indeed any other alteration of cellular immune function which could be attributed to the immunotherapy.

Studies on the ability of human glioma cells to elicit cytolytic lymphocyte responses in vitro have lead to several findings which may help to explain the failure of patients to make cytolytic lymphocyte responses to their own tumors in vivo. These studies have indicated that human glioma cells possess at least three mechanisms by which they may escape lymphocyte-mediated destruction: (1) low immunogenicity, perhaps related to a defect in their ability to stimulate helper T cells, (2) the secretion of immunosuppressive factor(s), and (3) the production of a protective mucopolysaccharide coat which impeded contact between lymphocytes and the glioma cells.

Five of 8 glioma lines tested were unable to stimulate allogeneic cytolytic lymphocyte responses in mixed lymphocyte-tumor cultures in vitro. However, in the case of 3 of the non-stimulatory glioma lines, cytolytic lymphocytes specific for the gliomas could be generated if responding lymphocytes were incubated with glioma cells in the presence of irradiated stimulator lymphocytes from a third individual. The specific cytolytic lymphocytes generated in this fashion were T cells, although nonspecific, non-T cytolytic cells, probably natural killer-like cells, are also generated in such cultures. The findings that these lines could not stimulate a cytolytic lymphocyte response in the absence of "help" from an allogeneic mixed lymphocyte reaction indicates that it may be of critical importance to learn how to modify such lines so as to increase their immunogenicity if they are to be used successfully as immunogens for the immunotherapy of glioma patients.

One of the non-stimulatory glioma lines, when incubated with responder and stimulator lymphocytes, inhibited the mixed lymphocyte reaction which would otherwise have occurred. Inhibition was observed regardless of whether the responder lymphocytes were from normal blood donors or from the glioma patient from whom the glioma line had been derived,

and was thus nonspecific in nature. Culture fluid from cultures of this glioma line were similarly inhibitory, whereas fluids from cultures of skin fibroblasts taken from the same patient did not contain immunosuppressive activity. The immunosuppressive substance(s) secreted by this glioma line has been shown to have a molecular weight greater than 10,000 daltons. Further biochemical characterization of this nonspecific immunosuppressive factor is in progress.

When two of the non-stimulatory glioma lines were incubated with lymphocytes, the lymphocytes were found to be prevented from making contact with the glioma cells by a clear "halo" surrounding the glioma cells. This halo can be removed by hyaluronidase and thus appears to represent a mucopolysaccharide coat produced by the glioma. In the case of one glioma line which has been studied extensively, removing this coat with hyaluronidase permits increased generation of cytotoxic lymphocytes specific for the glioma. However, such cytolytic lymphocyte generation requires the presence of both responder lymphocytes and third-party stimulator lymphocytes in the mixed lymphocyte-tumor cultures. Thus the inability of this glioma line to directly elicit cytolytic lymphocyte responses represents the combined effects of both the presence of a protective mucopolysaccharide coat and a separate defect in immunogenicity.

In parallel with the studies on the interactions between cytolytic lymphocytes and human glioma cells, we are employing a murine model to study the basic mechanism by which cytolytic lymphocytes lyse tumor cells. Use of the murine model is necessitated because these studies require populations of cytolytic lymphocytes having very high lytic activity. These studies have focused on the delineation of lymphocyte proteins involved in the generation or mechanism of action of cytolytic T lymphocytes. Initial studies led to the discovery of a protein called T11 which was shown to be present in the plasma membranes of a subpopulation of activated T lymphocyte blasts. Examination of the plasma membrane proteins of lymphocyte populations activated by several different means revealed a correlation between the presence of large amounts of T11 and the presence of cytolytic activity within the cell population. However, it is unknown whether T11 is found on cytolytic T cells or on helper T cells involved in the generation of cytolytic T cells. By using T cell growth factor we have generated five cloned lines of cytolytic T cells and two cloned lines of helper T cells. These lines will be examined for the presence of T11. Likewise we are attempting to elicit an antibody against T11 which will be used to study the possible function of T11 in the generation and/or action of cytolytic T lymphocytes.

Three patients have been treated with their own tumor cells under the immunotherapy protocol. They received serial injections of irradiated autologous cell suspensions and their humoral response was monitored. Antibody was undetectable in 2 of the patients prior to immunotherapy

and there was no change in their immune status when serial sera, which were drawn after treatment, were tested in the microcytotoxicity assay. The third patient had an initial positive response which soon became negative and was unchanged during immunotherapy.

#### PROPOSED COURSE:

The reorganized Surgical Neurology Branch is now in its third year and the bulk of the work of building the laboratory is complete. The in-depth and extended studies of glioma cell biology, chemotherapy/growth modulation testing, and humoral and cellular immunology are now fully active in all areas. For the area of glioma cell biology and characterization, major goals include: determination of the true heterogeneity of glioma cell populations through utilization of gel electrophoresis, immunological/fluorescent studies of glial fibrillary acid protein, fibronectin, and S-100 ganglioside analysis, flow cytometry, karyotyping growth characteristics, tumor angiogenesis factor and transmission, and scanning microscopy, automated image analysis and x-ray spectroscopy. The surface membrane properties of glioma cells with particular emphasis on the nature and immunological significance of the extracellular coat or matrix will continue to be pursued utilizing a combination of both immunological and electron microscopic techniques. Of great significance is the inability of lymphocytes to reach the glioma cell surface.

An area critical to the in vitro characterization effort is the detailed examination of the culture parameters critical to phenotypic expression in the cultures. Various parameters including confluency, migration from explant, surface charge and matrix composition, enzymatic dispersal, serum content and  $\text{Ca}^{2+}$  content are to be explored in detail for their effects on phenotypic expression utilizing a variety of specific and nonspecific markers. Cloning will be an increasingly important technique to answer questions of heterogeneity and phenotypic stability.

A new area of the characterization program, that of glioma metabolism, will be pursued vigorously, especially with respect to correlation with PECT scan 2-deoxyglucose data. For the further characterization of the metabolism of cultured astrocytomas, several other metabolites must be measured to better understand the basic properties of metabolism in cultured glial tumors. In order to better determine the energy status of the various grade of tumor, adenylate nucleotides (ATP, ADP, and AMP) and phosphocreatine will be measured. Measurements of cellular levels of GABA, glutamate, and glutamine, and the enzymes which interconvert these amino acids will yield important information on the metabolic fates of transported glutamate and glutamine. Long term plans include oxygen consumption studies of the various cell lines to determine if carbon sources other than glucose are oxidized. Lastly, the receptor systems for catecholamines will be further characterized by pharmacological means such as receptor binding, and antagonist/agonist studies.

Many critical questions remain in the verification of PECT scanning as a diagnostic tool. One problem that can be approached experimentally in cultured astrocytoma cell lines is the kinetic characterization of glucose and glucose analog uptake. We plan to determine the kinetic constants such as the  $K_m$  and  $V_{max}$  of glucose and glucose analog uptake, using previously published techniques (Cummins, Glover and Sellinger, J. Neurochem. 33, 779-785, 1979). We will also determine the glucose-6-phosphatase activity toward F-2-deoxy-d-glucose-6-phosphate. The measured kinetic constants and glucose-6-phosphatase may aid in the analysis of PECT scanning of tumors in situ.

A basic biological question of great importance in the use of tissue cultures as models for metabolism in situ is the stability over time of basic metabolic parameters. We are currently cloning several cell lines, one recently put in culture, and others which have been in culture for several months or several years. We will determine for each clone and for the wild type stocks, the rate of change over time of several selected metabolic characteristics, karyotype, and glial specific antigens. The lability/stability of these various characteristics will potentially provide critical information on the genetic or epigenetic basis of changes in characteristics in culture.

The nature, function and antigenicity of proteins released by glioma cells in culture will also be the subject of continued study. Techniques will include those already established in the laboratory and possible peptide mapping for any protein of special growth regulatory or immunological significance.

Radiobiologic studies of the available glioma cell populations will be added to the characterization program.

Finally, as the primate tumor model gliomas become available, characterization of these human JC virus-caused tumors will be pursued and a model chemotherapy system set up.

In the area of chemotherapy and biological modification, both the solid and aqueous phase microtiter assays will continue to be used extensively.

Drugs under active continued testing will include BCNU, PCNU, CCNU, AZQ, rapamycin, spirohydantoin, and phenytoin. The AZQ test series now includes 20 glioma derived cell lines and a larger series will be collected. Several new drugs will also be tested--cis-platinum (5 lines already tested), Henkel compound (highly toxic in two tests to date), methotrexate, and a pyrrolizine derivative. "Protective" compound WR 272 will also be tested with cis-platinum.

The phase II clinical study of the antiglioma efficacy of AZQ will continue. In addition, in collaboration with NCI, a new randomized 2-arm protocol (CCNU vs. AZQ) will be instituted. A new clinical



rating scale designed by SNB staff for assessment of glioma patients will be utilized in these studies. If indicated by promising in vitro studies, a clinical protocol for another of the new drugs may be instituted.

Studies of the quantitative predictive clinical value of the currently utilized microtiter tests will continue to be carried out. More data is required to determine the limits of these tests. The prospective study of in vitro planning of clinical chemotherapy will continue to collect data.

Mechanistic studies of nitrosoureas will also continue. The nuclear, cytoplasmic, and surface membrane properties responsible for sensitivity or resistance are beginning to emerge from current studies and should have important therapeutic implications. The strikingly different membrane response and cytoplasmic protein-binding patterns of sensitive and resistant glioma derived cell lines need to be explored in detail to define the cell biologic reasons for these differences. DNA alkylation repair will be pursued as tissue is available for study. With respect to possible alteration of therapeutic response, the role of intracellular sulphydryl content and the influence of the "differentiating" agents dibutyryl cyclic AMP and dimethylformamide will be tested with BCNU, AZQ, cis-platinum, spirohydantoin and Henkel compound. The use of liposomes for drug stabilization and delivery will be explored on a pilot basis.

Detailed mechanistic studies of AZQ, already underway, will receive increased attention. Since the mechanism of action of AZQ is not currently known, these are extremely important studies. Data already in hand indicate that nuclear and mitochondrial targets are likely.

For the immunology studies, the immunotherapy study protocol will continue to be a high priority. More patients will be entered. Several subgoals will continue to require strong effort in the coming year. These include: a) enhancement of tumor cell growth in tissue culture to provide adequate cell numbers for frequent immunotherapy; b) continued dissection of both the cellular and humoral aspects of the host response as well as the specificity of the antigenic properties responsible for eliciting the host response; c) increased baseline immune studies in protocol patients; and d) further toxicity/safety studies in nonhuman primates and rabbits.

In the area of humoral immunology dissection of the humoral response (antibody classes, antigen types) will continue as will the serial follow up of patient antiglioma antibody levels. Antigen purification will be crucial to the elucidation of basic immune response and also a key to the SNB programs of active immunotherapy. The present findings do not permit conclusions as to the antigenic categories being recognized, but the intimate relationship of cytotoxic antibody incidence with clinical course and the finding of a correlation between degree



of differentiation and the immune responses suggest that humoral immune factors may be important in the development of approaches to favorably alter the immune status of tumor patients.

With respect to the analysis of target cell factors in immune lysis the scanning and transmission studies of several parameters will be pursued. These will include presence or absence of extracellular matrix, membrane state, age in culture, and state of "differentiation" (i.e. after cAMP and DMF treatment). Radioisotopic quantification of membrane damage will be instituted. Special effort will be devoted to the question of modification of glioma derived cell antigenic expression since the present data indicate this to be a fruitful area.

During the coming year, work in cellular immunology will focus on further defining how the mechanisms by which glioma cells escape lymphocyte-mediated destruction operate and by what means they might be circumvented. We will attempt to define the lymphocyte subpopulations and lymphocyte products involved in mediating the allogeneic helper effect required for certain glioma lines to elicit cytolytic lymphocyte responses. This should lead to a better understanding of the nature of the defect in immunogenicity which such glioma lines possess. Attempts will be made to modify these glioma cells by chemical or enzymatic means so as to increase their immunogenicity. Glioma cells modified by these means will be tested for their ability to directly elicit cytolytic lymphocyte responses in the absence of any allogeneic helper effect.

The immunosuppressive substance produced by cultured glioma cells will be characterized biochemically. A number of cultured cell lines, both neoplastic and non-neoplastic, will then be screened for the production of similar immunosuppressive substance.

Likewise, we will examine the serum of glioma patients for the presence of this substance. If the production of this substance is unique to glioma cells, or at least to neoplastic cells, and is not simply a general property of cultured cell lines, further attempts will be made to purify the substance, to characterize its mechanism of action, and to produce an antiserum against it. Such an antiserum might have potential diagnostic and immunotherapeutic value.

While most of these studies will continue to involve the use of allogeneic combinations of lymphocytes and glioma cells, as our knowledge of how to circumvent the glioma cells' escape mechanisms increases, efforts to generate glioma-specific cytolytic lymphocytes using autologous combinations of lymphocytes and glioma cells will be intensified. If cytolytic lymphocytes specific for glioma antigens can be generated in autologous systems, we will then examine the ability of humoral antibody to effect the generation and action of glioma-specific cytolytic lymphocytes. Likewise, whether the action of such lymphocytes is HLA restricted will be studied.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 02368-03 SN								
PERIOD COVERED October 1, 1980 to September 30, 1981										
TITLE OF PROJECT (80 characters or less) <p style="text-align: center;">Biological and Immunological Factors in Peripheral Nerve Regeneration</p>										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: Barry H. Smith</td> <td style="width: 33%;">Deputy Chief</td> <td style="width: 16.5%;">SNB</td> <td style="width: 16.5%;">NINCDS</td> </tr> <tr> <td>Paul L. Kornblith</td> <td>Chief</td> <td>SNB</td> <td>NINCDS</td> </tr> </table>			PI: Barry H. Smith	Deputy Chief	SNB	NINCDS	Paul L. Kornblith	Chief	SNB	NINCDS
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SECTION Office of the Chief										
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CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS										
SUMMARY OF WORK (200 words or less - underline keywords) <p>             The cellular, biological and immunological factors in peripheral nerve regeneration are being studied in a rat vein-graft model. The vein graft serves as a chamber into which various biological agents such as collagen or "trophic" factors such as nerve growth factor as well as specific cell types grown in tissue culture can be added to study their effects on axonal regeneration. Quantitative light and electron microscopic measures are being utilized to analyze the effects. To date, certain human tumor cells in the vein-graft have enhanced regeneration whereas microcrystalline collagen has inhibited the process. The study of the influence of fetal dorsal root ganglion cells, cerebellar cells, cortical cells, and fibroblasts has continued and lectins have been added to study the role of glycoproteins in the regeneration process.           </p> <p>             A protocol (80-N-06) to study factors in failed human peripheral nerve regeneration has been in operation and the first patients have been entered and are currently under study.           </p>										

38 - SNB/IRP

## PROJECT DESCRIPTION:

I. OBJECTIVES: A study of the cellular and macromolecular factors influencing the success or failure of regeneration of axons after peripheral nerve injury in both a rat experimental model as well as in patients with peripheral nerve injuries.

## II. MATERIALS AND METHODS:

A. Nerve injury and graft: The left or right sciatic nerve in either an Osborne-Weber or Caesarian-delivered Fisher rat is transected sharply. Thereafter it is either 1) left alone for control; 2) reapproximated directly via a nerve graft with 9-0 nylon; 3) repaired with a 5 mm segment of vena cava obtained from a second animal of either the same or the other rat strain.

B. Vein graft placement: Under surgical microscopic control, the 5 mm segment of donor vein is sewn to the perineurium of the proximal and distal nerve ends with a total of four 8-0 nylon sutures. Care is taken to assure minimum tension.

C. Cell population for placement in graft: Rat or human cells are obtained from either the tissue culture stock lines in our laboratory or grown from new fetal cell cultures of the central nervous system as well as fibroblastic elements. Cultures from the different tissues as well as regions of the brain are quite distinct morphologically in tissue culture and maintain these characteristics over time. At the time for injection into the nerve graft, they are removed from the flask surface, centrifuged to provide a concentrated pellet and injected via a #27 needle into the graft.

D. Macromolecular factors: Microcrystalline collagen (nonantigenic) or other proteins such as nerve growth factor are placed within the vein graft prior to suturing under microscopic control to fill the vein graft cavity.

E. Lectins: In an effort to study the role of glycoproteins in the regeneration process, the proximal and/or distal nerve ends have been treated with various lectin concanavalin A (glucose) mannose, wheat germ agglutinin (N-acetylglucosamine), Ricinus communis agglutinin I and II-A (galactose and (N-acetylglucosamine), Ulex europaeus agglutinin (L-fucose), soybean agglutinin (N-acetylglucosamine) and Lotus tetragonolobus (fucose) have been applied to either stump for 1 or 2 hrs. prior to rupture of the nerve. Early regeneration at follow-up period of 1-4 weeks has then been studied by the techniques described below.

F. Follow-up periods: Animals so treated (see A-D above) are then followed for periods ranging from 0 time to 6 months prior to histologic study.



G. Histologic and ultrastructural examinations: All nerve and/or grafts are removed for study and cut into 1 mm segments to allow for precise reconstruction of the nerve. Fixation is accomplished with gluteraldehyde with standard Epon embedding. Thick sections are then cut for light microscope evaluation and thin sections are prepared for electron microscopy (uranyl acetate staining). Quantitation of numbers regenerating myelinated and unmyelinated axons is done by both light and EM methods.

H. Human peripheral nerve tissue and/or scans or neuromas have been obtained under Protocol No. 80-N-06. Two patients have undergone resection of neuromas resulting from failed or abnormal nerve regeneration. Cells derived from these neuromas have been studied in tissue culture and in the light and electron microscopes to determine the patterns of biological response to nerve injury that resulted in failed regeneration. Additional analysis has included clinical data (nerve conduction, evoked potentials, functional recovery) as well as histologic and ultrastructural examinations as per "F" above.

Human peripheral nerve cell cultures have been obtained from several patients with neurofibromatosis. The growth control mechanisms of these abnormal but non-malignant peripheral cells are also being studied.

### III. MAJOR FINDINGS:

Approximately 100 experiments have been completed to date with the rat sciatic nerve model. Major findings include (1) the inhibitory effects of heterologous and/or allogeneic grafts; (2) rapid loss of foreign (human glioma) cells from the vein graft but with an apparent enhancement of regeneration by certain tumor lines; (3) lack of significant growth promoting or inhibitory effects of various fetal rat cell lines tested to date; (4) inhibitory effect on regeneration of microcrystalline collagen placed within the rat sciatic nerve grafts; (5) striking inhibitory effects of various of the lectins tested to date.

For the patients with nerve injuries and neurofibromatosis currently under study, cultures from the injury patients have shown only very short-lived or no cell proliferation. The cultures derived from peripheral neurofibromas of the von Recklinghausen's disease patients have been successful in approximately 80% of cases and continued growth has not been a problem. Fibronectin and glial fibrillary acidic protein determinations will be utilized to determine the nature of the cells growing out. Scanning electron microscopy of both the post-traumatic neuromas and the neurofibromas (tumor and derived cells) is in process. The presence of antiglial antibodies is also being studied in both patient populations. Rather interestingly, the NF patients show a 40% positive response for antiglioma antibody. Much more work remains to be done on the human material.

## IV. SIGNIFICANCE:

Repair of damage to the nervous system is a major problem for basic and clinical neuroscience and the peripheral nerve injury and repair model under study here represent an approach to the study of this process. The project coincides with Institute interests as exemplified by the Stroke and Trauma Program. Improving nerve tissue regeneration is one of the highest priority areas in neurobiology today.

## V. PROPOSED COURSE:

The rat model studies will continue to be studied utilizing both light and electron microscopic techniques, including quantitative image analysis to determine the effects of the local environment on the regenerating axons. Use of growth factors will form a major part of the next effort as will use of plant lectins. Increased emphasis will be placed on patients with nerve injuries and neurofibromatosis to obtain tissue for culture and to study the different cell growth control mechanisms in these two populations. A collaborative arrangement has been made with a 700-patient NF clinic in Texas for additional tissue samples. For the nerve injury patients special emphasis will be placed on determination of the patterns of biological response that result in failed regeneration. Identification of the molecular signal or signals leading to initiation of Schwann cell and fibroblast proliferation after injury will also be a major issue.

## VI. PUBLICATIONS:

Smith, B. H. and Kornblith, P. L.: Axoplasmic transport in neurological surgery. Neurosurgery. (In press).

Smith, B. H.: Pathophysiology of axonal transport. In: Thoenen, H. and Kreutzberg, G. (Ed.): The Role of Fast Transport in the Nervous System. The Neurosciences Research Program Bulletin, 20, 1981. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02454-01 SN
PERIOD COVERED      October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Biological Studies of Human Pituitary Tumors		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	Paul L. Kornblith	Chief
Other:	Barry H. Smith Paul E. McKeever Craig Cummins Teodoro Dagli	Deputy Chief Neuropathologist Staff Fellow Guest Worker
		SNB      NINCDS SNB      NINCDS SNB      NINCDS Georgetown University
COOPERATING UNITS (if any)  Department of Neurosurgery, Georgetown University, Washington, D.C.		
LAB/BRANCH Surgical Neurology Branch, IRP		
SECTION: Office of the Chief		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.0	PROFESSIONAL: 1.0	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  Pituitary tumor cells in tissue culture have been determined in this laboratory to produce not only the hormones which they have been classically known to produce, but also a range of one to several other hormones. The purpose of this study is to explore the mechanisms which control the production of hormones by these tumor cells in an effort to improve pathological classification and diagnosis, accuracy of prognosis, prediction of recurrence, and ultimately therapeutic approaches. Cushing's disease is of special interest in this regard.		

## PROJECT DESCRIPTION

## I. OBJECTIVES:

The secretion of hormones by normal and neoplastic anterior pituitary cells has been an ongoing effort of this laboratory for several years. A surprising finding has been that certain pituitary tumor cells in tissue culture secrete not only the hormone which they have classically been known to produce clinically but also a range of other hormones. The explanation for this "dedifferentiated" behavior in molecular terms should provide insight into the abnormalities of the growth regulatory cells as well as to the clinical endocrinological picture they may present. In addition, studies of the variations of different pituitary tumor cell populations should be useful in elucidating the reasons why some tumors recur and/or are invasive, whereas others do not. Hormone secretion patterns are being utilized to provide diagnostic and prognostic evaluation of given tumors as well.

## II. MATERIALS AND METHODS:

A. Tissue culture technique: Pituitary tissue removed at surgery is placed in a medium consisting of F-10 with 10-12% fetal calf serum. The tissue removed at surgery is minced to 1 mm chunks and then explanted into plastic Falcon bottles with appropriate amounts of medium. For the assay of secreted hormones, the conditioned medium is saved and frozen at each medium change. After cellular outgrowth begins, the medium is changed on an approximately weekly basis. Cells are subcultured when required utilizing 0.25% trypsin.

B. Biological techniques: The detailed characterization of the pituitary cells requires performance of PPL0 or mycoplasma testing to determine that the cells are free of contamination. It also requires light and electron microscopy, biophysical and biochemical studies. The electron microscopic studies involve scanning, transmission, surface replica and x-ray spectrometry studies to evaluate the surface and intracellular characteristics of the tumor cells as well as normal pituitary. An automated image analysis system based on a Bausch and Lomb FAS II basic processing unit has been developed and put into use to provide quantitative measures of cellular and sub-cellular morphology as well as to surface mapping in various states.

C. Hormone screening: Routine screening of all cultured pituitary cells includes assays for ACTH, FSH, LH, GH, TSH and prolactin. Screening assays are done by Consolidated Biomedical Laboratories. A radioimmunoassay for prolactin is being set up in the SNB laboratories for detailed studies of the regulation of prolactin secretion. The influences of dopamine, bromocriptine,  $\text{Ca}^{++}$  on the regulation of prolactin secretion are being studied.

D. Neuropathological techniques: All standard neuropathological techniques for the evaluation of pituitary tumors are being employed



including hematoxylin and eosin, P-AB-PAS-OG stains, reticulin stains, and Alcian blue. In addition, immunohistochemical techniques are being employed. The problem of the proper diagnosis of Cushing's disease is being inspected with a combined clinical and neuropathologic approach. Certain cases of Cushing's disease of clinically proven pituitary origin do not respond to surgical removal of a solitary nodule. Immunohistochemistry using peroxidase to localize various pituitary hormones including ACTH shows multiple nodules of ACTH hyperplasia in some cases. Work is underway to diagnose such cases at first craniotomy so that proper removal can be undertaken at that time. Typically, resected portions of adenohypophysis are fixed in Zenker's and embedded in paraffin. Pilot sections of each block are cut and stained with H&E, P-AB-PAS-OG, or for reticulin. For peroxidase-anti-peroxidase (PAP) exploration of the tissue a procedure similar to that of Mason, et al (1969) is followed. Sections are cut serially at 5 $\mu$  and mounted onto glass slides using gelatin as an adhesive. The slides are deparaffinized and treated with iodine and Hypo, 5 and 3 minutes respectively, to remove fixative-based mercuric oxide crystals. This is followed by a 30-minute soak in 3% hydrogen peroxide in methanol to quench endogenous peroxidase. After rehydration the slides are soaked in 10% albumin in 0.05 M Tris-buffered saline (TBS) for 30 minutes, rinsed with TBS, and refrigerated overnight in a moist chamber with primary antisera directed against ACTH or other pituitary hormones. On the next day slides are drained of antisera, rinsed with 1% albumin in TBS, and exposed to a secondary antiserum (Goat anti-rabbit) for 30 minutes. Following this the slides are washed again and covered with peroxidase anti-peroxidase for 30 minutes to complete the immunoglobulin-enzyme bridge. To allow visualization of the peroxidase label, slides are then incubated for 10 minutes in a 30 mg/% solution of DAB (Sigma) with hydrogen peroxide. Following this reaction the slides are rinsed, counterstained with Mayer's hematoxylin, dehydrated, cleared, and mounted in Permount.

### III. MAJOR FINDINGS

Secreted hormone profiles over periods of time ranging from 1 week to several months have been obtained on some 10 human pituitary lines removed at surgery and grown in tissue culture. Hormones included in the assay have included ACT, GH, PRL, LH, and FSH. These assays have proven to have multiple values including the identification of particular tumor types when the pathology is not conclusive -- i.e. for the diagnosis of tumor types.

The pattern of hormone secretion observed in these pituitary lines has confirmed previous results in that many of the lines produce several hormones in addition to the one the pathology would have predicted. One line, however, which could not be identified on the basis of standard pituitary pathology was shown by hormonal assay to be a clear prolactinoma and not to secrete other hormones. Follow-up



of the patterns of hormonal secretion of these cell lines in culture over several weeks has shown a fall-off in secreted hormone production over this period. This decrease in hormonal production can, however, be partially reversed by administration of dibutyryl cyclic AMP. It seems quite possible that the analysis of pituitary tumor hormone secretion patterns will lead to a much more precise and meaningful diagnostic classification of pituitary tumors.

Aside from the diagnostic classification of pituitary tumors, the pattern of hormone secretion yields the opportunity to study the regulation of phenotypic expression in neoplastic pituitary cells. This, of course, has implications for determining both the state of differentiation of each pituitary (and hence its malignant potential) as well as the nature of the abnormal growth control mechanisms. This area has been pursued over the past year and will remain the subject of major continued effort. The goals remain the determination of the malignant biological potential of a given tumor line (and hence prediction of clinical course) as well as the development of biological means of controlling abnormal pituitary cell growth in patients.

A third major area of interest for pituitary cells in culture is the study of the mechanisms and regulation of the secretion of pituitary hormones. The in vitro system is especially useful for the analysis of these cellular mechanisms. In studies completed to date dibutyryl cyclic AMP appears to have a regulatory role in the synthesis and/or secretion of luteinizing hormone and follicle stimulating hormone, but not ACTH, GH, or prolactin to any significant degree. Receptor-coupled dopaminergic regulation of prolactin secretion is also under study utilizing bromocriptine. With the recent arrival of Dr. Craig Cummins, who is an expert in receptor biochemistry, this area will be the subject of increased effort in the coming year.

A case of refractory Cushing's disease was studied in detail this year under the direction of Dr. Paul McKeever. The patient had pituitary-dependent hypercortisolism which did not respond to partial resection of the anterior lobe. Histochemical stains showed multiple focal expansions of fibrovascular stroma by cells. Gradual and abrupt transitions between normal and expanded acini occurred. Immunoperoxidase staining for ACTH and other pituitary hormones revealed that these multiple foci contained ACTH positive cells. Less than 10% of the cells in these foci were negative for ACTH and positive for other hormones. Serial sectioning and immunohistochemical staining showed that the focal expansions of predominantly ACTH producing acini (were/were not) connected with each other. Combined morphologic and immunohistochemical data provide evidence that ACTH-cell hyperplasia caused the Cushing's disease in this case.

## IV. PROPOSED COURSE

A major goal is the study of the basic biochemistry and pharmacology of pituitary secretion. A human pituitary cell line which has been shown to secrete significant amounts of prolactin has recently been acquired. Prolactin secretion will be examined in this line. While some details of the regulation of prolactin synthesis are known for rodent pituitary cultures, little is known about the regulation in human pituitary cultures. In rodent pituitary, hypothalamic inhibiting factors, dopamine (by a cyclic AMP dependent mechanism) have all been implicated in the inhibitory regulation of prolactin synthesis and release. We intend to assay the binding characteristics of both dopamine and ergot alkaloids, and simultaneously measure cyclic AMP and prolactin synthesis and release. Electron microscopic findings will be correlated as well. With this first step we hope to gain some appreciation of the basic mechanism of human prolactin synthesis and release. Other lines will also be examined as they become available. Gradually the study will be expanded for other hormones also.

Routine neuropathologic and hormonal screening of human pituitary tumors will continue to be carried out. Evident from the study of refractory Cushing's disease secondary to ACTH-cell hyperplasia is the value of establishing the diagnosis of hyperplasia or adenoma at surgery. Accordingly, effort will be concentrated on developing a one-step method for characterizing ACTH secreting cells in sections of human anterior pituitary. This method will be used to distinguish between abnormal, hyperplastic, and normal tissue biopsies taken during transphenoidal hypophysectomies and craniotomies.

Finally, attention will be directed toward the question of determination of the potential for malignancy, invasiveness, or recurrence or individual human pituitary cell lines. The extent to which the pattern of hormonal secretion is indicative of relative differentiation and and hence of the relative benignity or malignancy of the pituitary cells will be assessed.

## V. PUBLICATIONS:

None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 01047-19 SN
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Radionuclide Ventriculography and Cisternography		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: G. DiChiro  OTHER: G.S. Johnston A.E. Jones R.A. Boooks	Chief, Neuroradiology and Computed Tomography Section  Chief, Nuclear Medicine Dept. Assistant Chief Staff Physicist	SN NINCDS  NM CC NM CC SN NINCDS
COOPERATING UNITS (if any)  Nuclear Medicine, Clinical Center, NIH		
LAB/BRANCH Surgical Neurology Branch		
SECTION Neuroradiology and Computed Tomography Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.083	PROFESSIONAL: 0.083	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input checked="" type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  <u>Radionuclide ventriculography</u> and <u>cisternography</u> are diagnostic tools permitting the morphologic and dynamic study of the cerebrospinal fluid pathways more accurately than has ever been possible with any other diagnostic test.  The adjunction of <u>positron emission computed tomography</u> to our diagnostic armamentarium should improve significantly the information content of our radionuclide ventriculograms and cisternograms.		

Objectives: A gamma emitting isotope injected within the cerebrospinal fluid pathways will permit in subsequent head scans the pictorial outline of the ventricular system (isotope or radionuclide ventriculography) and of the subarachnoid intracranial spaces (isotope or radionuclide cisternography). Information about the anatomical status of the cerebrospinal fluid cavities, and, by multiple serial scans, of the normal and abnormal dynamics of the cerebrospinal fluid itself will be obtained. The spinal CSF spaces may also be evaluated.

Methods Employed: The radionuclide cisternography and ventriculography procedures are now well established.

Recently we have devoted particular attention to one aspect of the CSF flow, i.e., its descent to the spinal subarachnoid space.

Major Findings: We have initiated positron emission computed tomography (PECT) of the CSF cavities after intraventricular (IV ventricle) introduction of a positron emitter ( $^{68}\text{GaEDTA}$ ) in primates.

Significance to Bio-Medical Research and the Program of the Institute: Legions of authors are studying this remarkable fluid (CSF) which still remains in many respects uncomprehended since Cotugno first described it in 1764. In particular we now have a diagnostic tool to gather information about the "terra incognita" which is represented by the basal and convexity subarachnoid pathways, as well as the spinal CSF compartment. In this area, the CSF spinal descent studies should enable us to determine what is the importance of the spinal CSF route of flow as an alternative pathway of resorption. The observations of the spinal descent pattern of the CSF have also heuristic significance in regard to a possible analysis of metabolites and drugs distribution through the CSF from the endocranial cavity to the easily accessible spinal theca.

Proposed Course of Project: Further information about the normal and abnormal cerebrospinal fluid cavities, and the normal and pathologic flow of CSF will be gathered by the techniques of radionuclide cisternography and ventriculography. The adjunction of the capabilities for PECT (an ORTEC-ECAT, PECT device has been installed and is operational at the NIH Clinical Center) permits significant refinements in the techniques of radionuclide cisternography and ventriculography. In particular, the use of radiopharmaceuticals tagged with positron emitters (e.g., chelating substances labeled with  $^{68}\text{Ga}$ ) allows for a better demonstration of the tagged CSF in the deep CSF cavities. This improved demonstration is possible through the tomographic display of images representing axial transverse slices. The problem of the superimposition of the radioactivity in the superficial tissues, so disturbing in the interpretation of conventional radionuclide CSF scinti-photographic studies, is practically eliminated. The NIH Neuro-PET, which is presently being built will offer soon further resolution capabilities of the CSF cavities with PECT.

Publications: None



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  201 NS 01195-17 SN
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Radiographic and Radioisotopic Angiography of the Spinal Cord		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	G. DiChiro	Chief, Neuroradiology and Computed Tomography Section
		SN NINCDS
OTHER:	J.L. Doppman	Chief
	K.G. Rieth	Staff Radiologist
	P.L. Kornblith	Chief
	E. Quindlen	Senior Staff Fellow
	G.S. Johnston	Chief
	A.E. Jones	Assistant Chief
		DR CC DR CC SN NINCDS SN NINCDS NM CC NM CC
COOPERATING UNITS (if any) Diagnostic Radiology and Nuclear Medicine Departments, Clinical Center, NIH; Medical Examiner's Office, Department of Public Health, Philadelphia, PA.		
LAB/BRANCH Surgical Neurology Branch		
SECTION Neuroradiology and Computed Tomography Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
0.083	0.083	
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input checked="" type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  <u>Selective arteriography</u> (radiographic) of the spinal cord is a diagnostic technique which has proven to be very informative in cases of arteriovenous malformation, tumor, obstructive vascular disease, trauma, and postradiation damage of the spinal cord.  <u>Radioisotope angiography of the spinal cord</u> offers distinct advantages as a screening method, and in certain types of intraspinal pathology may give information not available by any other diagnostic test.  <u>Preliminary experience with computed tomography of the spine after injection of contrast medium</u> indicates that this methodology is useful in the evaluation of certain vascular lesions of the spinal cord.		



## Project Description:

Objectives: The introduction of cerebral angiography (1927) has markedly increased our knowledge of the vascular pathology of the brain. The vascular pathology of the spinal cord, on the other hand, still remains a largely unexplored area.

Since 1964 we have been carrying out angiographic studies of the spinal cord and developed this technique into a reliable diagnostic tool. Selective injection of the contrast medium has made the difference between an occasional demonstration, and the consistent visualization of the spinal cord vasculature.

The usefulness of selective arteriography in cases of spinal cord arteriovenous malformations is now well established. We are continuing to use this technique to:

- 1) Learn more about the pathophysiology of the spinal cord arteriovenous malformations so that a better treatment of these important and frequent lesions may be developed.
- 2) Evaluate how useful spinal cord angiography is in cases of spinal cord tumors.
- 3) Establish whether or not this technique can be of diagnostic value in the study of obstructive spinal cord vascular disease.
- 4) Assess the usefulness of this technique in intervertebral disc pathology.
- 5) Evaluate the diagnostic possibilities of this procedure in post-traumatic spinal cord injury with or without vertebral fractures.
6. Establish the value and limits of newly introduced radioisotopic angiography of the spinal cord.
- 7) Explore the possible emergency therapeutic means which could be employed to treat and cure, or at least minimize the effects of the dreadful postangiographic cord complications.
- 8) Acquire new information regarding the fine vasculature of the human spinal cord, with particular emphasis on the intrinsic vessels (sulcal and central arteries and other perforating or penetrating branches).

This goal is accomplished by post-mortem microangiographic techniques in cadavers of all age groups. We are paying particular attention to cords of aged adults.

Methods Employed: Selective arteriograms with modern catheter techniques are carried out in patients in whom spinal cord vascular or tumoral lesions are suspected. Rapid serialograms, subtraction and magnification are used to better visualize the injected vessels.

For the technique of radioisotope angiography of the spinal cord a bolus of 15 mCi of  $^{99m}\text{Tc}$  human serum albumin or  $^{99m}\text{TcO}_4^-$  is injected in a left antecubital vein. Immediately afterwards, scintiphotographic rapid flow studies of the various segments of the spine are obtained with a scintillation camera. Our scintiphotographic data have been significantly ameliorated by a computer assisted analysis and reconstruction of images, as well as by isometric contour computer display of the data.

For the technique of computed tomographic angiography of the spinal cord we use a computed tomography (CT) body scanner and we carry out timed serial tomograms of the area of interest of the spine after the intra-venous introduction of a bolus of angiographic contrast medium.

For the post-mortem studies of the vessels of the human spinal cords, (aged adults) we have used our previously developed microangiographic techniques.

Based on the observation made elsewhere, that in two patients who died soon after aortography with spinal cord complications, the iodine content in the CSF was enormously increased, we have been attempting an emergency therapeutic method consisting of flushing out (lavage) the "iodine contaminated" CSF.

Major Findings: We have continued to accumulate experience in the areas of :

- 1) Selective arteriography in vascular malformations of the spinal cord.
- 2) Selective arteriography in cases of herniation of thoracic discs.
- 3) Post-mortem microangiographic evaluation of the aged human cord.
- 4) CSF lavage in patients who develop symptoms and signs of cord involvement after abdominal aortography or other types of arteriographic studies.

Significance to Bio-Medical Research and the Program of the Institute: Radiographic and radioisotopic angiography of the spinal cord are increasing our understanding of the large group of conditions in which vascular lesions of the cord represent the basic pathologic element.

Proposed Course of Project: Post-mortem microangiography of the aged adults' cords should offer new insights on such conditions as obstructive vascular disease of the cord due to arteriosclerosis and cervical spondylosis, and possibly on degenerative and demyelinating cord diseases.

We are "watching" for possible further technical developments of the technique of selective arteriography of the spinal cord. We are considering initiating the use of angiotomography for a better visualization of the smaller vessels, possibly the intrinsic arteries and veins of the cord.

Improved x-ray vascular contrast media will also enhance the diagnostic possibilities of spinal cord angiography. We are following very closely the recent developments in the area of polymeric, ion-balanced and non-ionic iodinated x-ray contrast media.

Radioisotope angiography of the spinal cord is a method which we have been using as a screening and follow-up procedure.

Computed tomographic (transmission) angiography of the spine and spinal cord represents one of the areas in which we will concentrate a great deal of interest.

We have some expectation that positron emission computed tomography particularly with the use of the high resolution Neuro-PET (designed in our Section), will allow us to study blood flow and metabolism (glucose metabolic rate) of the cord in a non-invasive fashion.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 NS 01654-14 SN
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Experimental Spinal Cord Angiography		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	G. DiChiro	Chief, Neuroradiology Computed Tomography Section
SN	NINCDS	
OTHER:	E. Quindlen P.L. Kornblith B.H. Smith	Senior Staff Fellow Chief Senior Staff Physician
SN	NINCDS NINCDS NINCDS	
COOPERATING UNITS (if any) J. Fein, Department of Neurological Surgery, Albert Einstein College of Medicine, Bronx, NY, formerly of Armed Forces Radiobiology Research Institute, Bethesda, MD; K. Earle, Chairman, American Registry of Pathologists, Washington, DC.		
LAB/BRANCH Surgical Neurology Branch		
SECTION Neuroradiology and Computed Tomography Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:  0.083	PROFESSIONAL:  0.083	OTHER:  0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) <u>Experimental spinal cord angiography</u> in the <u>rhesus monkey</u> is increasing our understanding of the blood supply of the spinal cord both in physiological and pathological conditions. Recently this technique has been used as a basis for attempts at experimental surgical revascularization of the cord in primates.		

Objectives: The clinical value of the NIH-developed technique of selective arteriography in the management of arteriovenous malformations and tumors (in particular hemangioblastomas) of the spinal cord is now well established.

In order to expand the clinical applications of arteriography of the spinal cord we are working with experimental angiographic and microangiographic models in primates.

Previously, we have concentrated our attention on the area of experimental obstructive vascular disease of the spinal cord in the rhesus monkey. More recently much of our experimental investigation has dealt with a catastrophic iatrogenic pathological condition, postradiation myelomalacia (myelitis), which occurs more frequently than is generally realized. In this area we are trying to establish whether the basic pathological lesion of this dreadful complication is primarily neurogenic or vascular.

In this fiscal year we have initiated the use of spinal cord arteriography in primates as an indispensable prerequisite to attempts at revascularization of the thoraco-lumbar cord.

Methods Employed: Preradiation angiographic studies (selective technique) of the thoracolumbar segment of the spinal cord are carried out in young, healthy rhesus monkeys. Soon after, selective irradiation of the thoracolumbar cord using a LINAC accelerator is initiated. Total dosage and modalities of delivery are chosen to approximate the radiation protocol which most often seems to cause myelomalacia in human patients. At the end of the radiation, the monkeys are kept under careful observation for periods of many months. Neurological testing of the lower limbs is performed twice a week. If and when the monkeys show signs of developing or established paraplegia, repeat selective arteriography of the irradiated segment is carried out. Following this, the animals are perfused for microangiography of the spinal cord and then sacrificed. The cord is studied by gross observation, microangiography, routine histology and special myelin stains. Careful gross and histological analysis of the neighboring aortic segment, its branches and the pertinent radiculomedullary arteries is also carried out.

For the cord revascularization project we carry out presurgical spinal cord arteriography in the rhesus. In this primate the arteria radiculomedullaris magna (artery of Adamkiewicz) most frequently originates from the second left lumbar artery. After angiography has provided us the basic information on the cord vascularization, we perform surgery to anastomose one of the last intercostal arteries (XI, XII) with the anterior spinal artery below the artery of Adamkiewicz inosculation. For this purpose a transversectomy and partial resection of one vertebral body is necessary. The anastomosis is end-to-side with the help of the surgical microscope. If and when we will be able to accomplish successful anastomoses, the artery of Adamkiewicz and the anterior spinal artery will be ligated at the point of their inosculation. As we have proven in previous studies (Fried, L.C., Di Chiro, G. and Doppman, J.L. Ligation of major thoraco-lumbar spinal cord



arteries in monkeys. J. Neurosurg. 31:608-614, 1969), surgical devascularization of this type causes paraplegia in normal monkeys. In the "revascularized" monkey, this should not be the case because of the additional blood supply provided by the anastomotic intercostal artery.

Major Findings: We are on the course of evaluating the pathological changes of the spinal cord from monkeys in which we successfully induced postradiation paraplegia (myelopathy). Our efforts with experimental cord revascularization in primates have just begun.

Significance to Bio-Medical Research and the Program of the Institute: We should be able to shed some light on the pathogenesis of the postradiation myelitis. This is not a rare complication in human patients (over 500 cases have been reported in literature). The implication of a successful revascularization of the cord for the many human patients suffering from ischemic cord diseases are obvious.

Proposed Course of Project: Appraisal of the postradiation data which we have already collected as well as new data in other irradiated animals now under observation. We will attempt to study (by angiography and micro-angiography) human patients (or human specimens) with postradiation spinal cord damage. Our effort with the cord revascularization project will proceed.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02073-08 SN
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Computed Tomography (Transmission)		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	G. DiChiro  R.A. Brooks K.G. Rieth V.J. Sank	Chief, Neuroradiology and Computed Tomography Section Staff Physicist Staff Radiologist Expert  SN NINCDS SN NINCDS DR CC SN NINCDS
OTHER:	P.L. Kornblith B.H. Smith E. Quindlen A.M. Cormack J.L. Sever W.T. London	Chief Senior Staff Physician Senior Staff Fellow Physicist Chief Chief, Experimental Pathology Section  SN NINCDS SN NINCDS SN NINCDS Tufts Univ. ID NINCDS ID NINCDS
COOPERATING UNITS (if any) Diagnostic Radiology, Nuclear Medicine Department, CC, NIH; Infectious Diseases Branch, IRP, NINCDS, NIH; Physics Department, Tufts University, Medford, MA.		
LAB/BRANCH Surgical Neurology Branch		
SECTION Neuroradiology and Computed Tomography Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.58	PROFESSIONAL: 1.58	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input checked="" type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) <u>Computed Tomography (CT)</u> - in its transmission and emission modalities, represents the main research area of the Neuroradiology & Computed Tomography Section.  Ongoing clinical - animal/experimental research projects in transmission CT include studies of degenerative, demyelinating and atrophic processes of the brain, hydrocephalus, brain edema, postradiation cerebral necrosis, surgically correctable lesions in young patients affected by chronic epilepsy, diseases of the spine and the spinal cord, attempts at tissue characterization of normal and abnormal (e.g. tumoral) cerebral tissue, and an experimental glioma model in primates.  Physics projects: improved dual-energy CT scanning using both a split-detector and a dual kVp method; analysis of aliasing effects and development of methods for their elimination; phantom studies for the evaluation of artifacts and calibration of CT machines; feasibility tests for a new type of CT device which will use <u>protons instead of x-rays</u> .		

Objectives: To advance the clinical applications of CT. Attenuation is being devoted to trying to improve the resolution of the CT devices. Advanced quantitative assessment of the attenuation values (profiles, regions of interest) are used in an attempt to improve the diagnostic specificity of CT; the goal is to enhance our capability of distinguishing between different types of lesions which present with similar qualitative findings. Differentiation of the various tissues' chemical components (tomochemistry) through dual-energy scanning or, possibly in the future, by means of proton CT, is a promising line of our research. An experimental glioma model in primates offers great hopes for improvement of our knowledge of the optimal parameters for CT scanning of the brain and also for enhancing our ability to differentiate between the various glioma types.

Methods Employed: Clinical CT neuro-scanning is now a standard diagnostic procedure. Groups of patients with various disease conditions are studied by CT of the brain and/or spine-spinal cord.

A split detector of original design has been built to carry out simultaneous dual-energy scanning.

An experimental (virus induced) glioma model in primates has been developed and tested and is being used for a variety of purposes.

Basic experiments being carried out as a preliminary step to build the PROTO-Scanner, involve determination in various phantoms of the absorption coefficient of the proton beam produced by a cyclotron. The phantoms include organic materials (particularly organic solutions of various concentrations).

Major Findings: In the clinical area we have:

1) Continued to accumulate experience in the area of CT of white matter degenerative processes. We have described a new, characteristic type of CT pattern in adrenoleukodystrophy.

2) Dual-energy CT (Tomochemistry) - A significant amount of dual-energy CT data on patients has been accumulated. Our goal is to establish dual-energy CT "signature" of the various tissues. With this technique, the CT recognition of even minimal amounts of certain tissues (particularly fat) as well as electrolytic fluids (CSF) is greatly improved as compared to conventional CT. The split detector developed in our Section for the purpose of dual-energy scanning has been helpful in these studies.

3) Our CT research on the spine and spinal cord has continued.

4) Observed interesting findings concerning postradiation necrosis of the brain. These findings may mimic brain tumors (recurrence or spread). Their recognition, therefore, is of capital importance.

5) Analyzed a large group of patients affected by chronic epilepsy to determine how frequently surgically correctable epileptogenic lesions can be detected solely by CT.

In the animal experimental area we have:

1) Completed a CT study on the edema in primates. Cryogenically induced cerebral edema in the rhesus monkey was analyzed by serial CT scans in both axial and coronal planes. The outset, progression (peak at the fourth - fifth day) and resolution of the vasogenic cerebral edema have been assessed. An attempt was made to correlate the low CT attenuation values of the involved areas with the specific gravity of corresponding fresh edematous brain specimens.

2) In tandem with our clinical activity connected with the differentiation of the various types and stages of hydrocephalus, we have been performing experimental studies in primates with a variety of obstructive hydrocephalus models to evaluate timing of appearance and evolution of the periventricular hypodensity (thought to be related to the transependymal passage of CSF).

3) In tandem with our clinical activity on the spinal cord and spinal CSF we have carried out experiments in primates trying to develop better visualization of the cord and the spinal CSF through intravenous enhancement (intravenous CT myelography). From preliminary observations, it would appear that possibly there is an early relative enhancement of the cord followed by late relative enhancement of the CSF. By exploiting these features one could extract valuable additional information from CT of the spine after simple intravenous injection of contrast medium and, thus, avoid the intrathecal administration.

4) Continued to work with CT in a model of experimental (virus-induced) glioma in primates.

Significance to Bio-Medical Research and the Program of the Institute: The diagnostic abilities in the area of neuroradiological disease are fundamentally altered by the introduction of CT. The progress in this area is fast. Statements regarding the future significance of this methodology could be surpassed and rendered obsolete in a short time.

Proposed Course of Project: In the Neuroradiology and Computed Tomography Section, CT will be the main area of research for years to come. We will proceed with a multipronged approach: 1) clinical work on the brain, sella turcica-pituitary gland (microadenomas), spinal cord and eye; 2) experimental research on primates; 3) tomochemistry of the CNS; 4) theory (mathematics, physics); 5) planning and building a new type of CT device (using protons rather than x-rays).

A new journal "JOURNAL OF COMPUTER ASSISTED TOMOGRAPHY" originates from this Section (Eds. Di Chiro and Brooks).

Publications: Di Chiro, G.: Improvement in computed tomography spatial resolution. Computerized Tomography. Ed: J.M. Caille and G. Salamon, Pub: Springer-Verlag, pp 12-15, Berlin, 1980.

Di Chiro, G., Eiben, R.M., Manz, H.J., Jacobs, I.B. and Schellinger, D.: A new CT pattern in adrenoleukodystrophy. Radiology 137:687-692, 1980.

Brooks, R.A., Di Chiro, G., and Keller, M.R.: Explanation of cerebral white-gray contrast in computed tomography. J. Comput. Assist. Tomogr. 4:489-491, 1980.

Sheridan, W.T., Keller, M.R., O'Connor, C.M., Brooks, R.A. and Hanson, K.M.: Evaluation of edge-induced artifacts in computed tomography scanners. Med. Phys. 7:108-111, 1980.

Keller, M.R., Kessler, R.M., Brooks, R.A. and Kirkland, L.R.: Optimum energy for performing CT iodinated contrast studies. Brit. J. Radiol. 53:576-579, 1980.

Brooks, R.A., Sheridan, W.T., Keller, M.R., and O'Connor, C.M.: Progress toward quantitative computed tomography. IEEE Trans. Biomed. Eng. 27:1121-1127, 1980.

Brooks, R.A., Di Chiro, G., Mitchell, L.G., and O'Connor, C.M.: On the relationship between computed tomography number and specific gravity. Phys. Med. Biol. 26:141-147, 1981.

Brooks, R.A.: Comparative evaluation of CT scanner technology. In: Fullerton, G.D and Zagzebski, J.A. (Eds.): Medical Physics of CT and Ultrasound: Tissue Imaging and Characterization. New York, American Institute of Physics, 1980, pp. 53-69.

Brooks, R.A.: Computational principles of transmission CT. In: Fullerton, G.D. and Zagzebski, J.A. (Eds.) Medical Physics of CT and Ultrasound: Tissue Imaging and Characterization. New York, American Institute of Physics, 1980, pp. 37-52.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (00 NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02315-04 SN																																												
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TITLE OF PROJECT (80 characters or less)  Positron Emission Computed Tomography																																														
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<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 40%;">G. DiChiro</td> <td style="width: 40%;">Chief, Neuroradiology and Computed Tomography Section</td> <td style="width: 10%;">SN NINCDS</td> </tr> <tr> <td></td> <td>R.A. Brooks</td> <td>Staff Physicist</td> <td>SN NINCDS</td> </tr> <tr> <td></td> <td>V.J. Sank</td> <td>Expert</td> <td>SN NINCDS</td> </tr> <tr> <td></td> <td>R.L. DeLaPaz</td> <td>Guest Staff Fellow</td> <td>SN NINCDS</td> </tr> <tr> <td></td> <td>N.J. Patronas</td> <td>Guest Staff Fellow</td> <td>SN NINCDS</td> </tr> <tr> <td></td> <td>P.L. Kornblith</td> <td>Chief</td> <td>SN NINCDS</td> </tr> <tr> <td></td> <td>B.H. Smith</td> <td>Senior Staff Physician</td> <td>SN NINCDS</td> </tr> <tr> <td></td> <td>E. Quindlen</td> <td>Senior Staff Fellow</td> <td>SN NINCDS</td> </tr> <tr> <td></td> <td>R.J. Porter</td> <td>Act'g Chief, CES</td> <td>ET NINCDS</td> </tr> <tr> <td></td> <td>M.E. Newmark</td> <td>Neurologist</td> <td>EB NINCDS</td> </tr> <tr> <td></td> <td>T.N. Chase</td> <td>Director</td> <td>IRP NINCDS</td> </tr> </table>			PI:	G. DiChiro	Chief, Neuroradiology and Computed Tomography Section	SN NINCDS		R.A. Brooks	Staff Physicist	SN NINCDS		V.J. Sank	Expert	SN NINCDS		R.L. DeLaPaz	Guest Staff Fellow	SN NINCDS		N.J. Patronas	Guest Staff Fellow	SN NINCDS		P.L. Kornblith	Chief	SN NINCDS		B.H. Smith	Senior Staff Physician	SN NINCDS		E. Quindlen	Senior Staff Fellow	SN NINCDS		R.J. Porter	Act'g Chief, CES	ET NINCDS		M.E. Newmark	Neurologist	EB NINCDS		T.N. Chase	Director	IRP NINCDS
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COOPERATING UNITS (if any) BEIB, DRS, NIH; Naval Res. Lab., Wash., DC, Wash. Univ., St. Louis, MO; Lab of Cerebral Metabolism, NIMH, NIH; ODIR, NINCDS; EB, NINCDS; ETB, NINCDS; LCP, NCI; Brookhaven National Lab., Upton, NY; Univ. of Wash., Seattle, WA; Div. of Nucl. Med., Dept. of Rad. Sciences, UCLS, Los Angeles, CA.																																														
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SUMMARY OF WORK (200 words or less - underline keywords)  <u>Positron Emission Computed Tomography (PECT)</u> allows us to obtain pictorial data (e.g. axial transverse or coronal images of the brain) as well as dynamic functional data (such as regional cerebral glucose consumption rate); measurements of the storage, degradation and turnover of tagged metabolites; follow-through of the movement of the CSF in the deep CSF intracranial cavities). The unique property of PECT is that it provides physiologic information not available with any other imaging procedure.  During the last year significant progress has been made in our Section on the construction of a high-resolution high-sensitivity scanner for head and animal studies -- the Neuro-PET.																																														

## Names, Laboratory and Institute Affiliations, and Titles of Principal Investigators and All Other Professional Personnel Engaged on the Project

Continued:

G.S. Johnston	Chief	NM CC
A.E. Jones	Assistant Chief	NM CC
R.M. Kessler	Staff Physician	NM CC
R.G. Blasberg	Medical Officer	LCP NCI
A.P. Wolf	Senior Chemist	Brookhaven
S. Larson	Associate Professor	Univ. of Washington

OTHER:	L. Sokoloff	Chief	LCM NIMH
	D.E. Kuhl		UCLA
	M.E. Phelps		UCLA

## Project Description:

Objectives: Recent new developments have made a significant difference in the practical and clinical application of PECT. The two most important of these developments are: 1) efficient PECT devices (scanners) have been developed and some are commercially available, and 2) the original Sokoloff's autoradiographic technique for determining local glucose metabolic rate in experimental animals has been converted into a PECT method for living human subjects (see below).

Methods Employed: The radioactivity originating from a positron emitting radionuclide, which generally is introduced by the intravenous route, is detected plane by plane (tomography) with axial-transverse (horizontal) or coronal incidence, and the images of this distribution in slices or cuts through the body area of interest are produced, displayed and recorded in a variety of fashions. The most interesting radionuclide is at present  $^{18}\text{F}$ -2-deoxyglucose ( $^{18}\text{FDG}$ ). The application of this tracer is a direct derivation of the original, NIH-developed, Sokoloff's autoradiographic technique in experimental animals. In living human patients, it is now possible with  $^{18}\text{F}$  (110 minute half-life) tagged 2-deoxyglucose to obtain pictorial data (axial transverse and coronal images) as well as quantitation of the local cerebral glucose metabolic rate. Other positron emitting radiopharmaceuticals of interest are  $^{68}\text{Ga}$  chelated tracers ( $^{68}\text{Ga}$  is generator produced and has a 68 minute half-life); the  $^{68}\text{Ga}$  tracers would be particularly interesting for the analysis of the CSF circulation (PECT cisternography). The use of tracers tagged with the short-lived  $^{11}\text{C}$ ,  $^{13}\text{N}$  and  $^{15}\text{O}$  will require a cyclotron on the NIH premises.

Major Findings:

1) The protocol project " $^{18}\text{F}$ -2-Fluoro-2-deoxy-D-glucose (FDG) Positron Emission Computed Tomography (PECT) in Typing of Cerebral Gliomas" is well under way with the study of our first group of patients with cerebral glioma.

Glucose metabolism in human cerebral gliomas has been studied at the NIH using  $^{18}\text{F}$ -2-fluoro-2-deoxy-D-glucose ( $^{18}\text{FDG}$ ) and positron emission computed tomography (PECT). It is known that in some non-CNS experimental

tumors a higher histologic grade of malignancy is associated with an increased rate of glycolysis. The corollary increase in hexokinase activity and decrease in glucose-6-phosphatase activity with increased growth rate noted in biochemical studies of these tumors provides a situation conducive to determination of grade of malignancy by measurement of "trapped" 2-deoxy-D-glucose-6-phosphate (Weber, G. [1977]: N. Engl. J. Med., 296:486; Sokoloff, L., Reivich, M., Kennedy, C., Des Rossiers, M., Patlak, C., Pettigrew, K., Sakurada, O., Shinohara, M. (1977): J. Neurochem. 28:897). In vitro biochemical and histochemical studies suggest that glycolysis, unlike oxidative metabolism, is also positively correlated with histologic grade of malignancy in human and animal gliomas (Wollemann, M. [1972]: In: Handbook of Neurochemistry, edited by A. Lajtha, p. 503, Plenum Press, New York). One histochemical study of human glioma tissue associates higher grades of malignancy with increased hexokinase activity and decreased glucose-6-phosphatase activity (Perria, L., Viale, G., Ibba, F., Andreussi, L., and Viale, E. Neuropsychiatria 20:3, 1964).

A positive correlation between isotope activity of trapped  $^{18}\text{F}$ FDG-6-P measured by PECT (ECAT II scanner) and histologic grade of gliomas is suggested by our patient scans to date. Sixteen studies on 13 patients were done between May, 1980 and January, 1981, and are summarized in Table I. Local cerebral metabolic rate for glucose (LCMRGlc) values for high grade tumors generally fall in the range of 6 to 12 mg/100gm/min and values for low grade tumors are in the range of 3 to 6 mg/100gm/min in our scans. However, due to the fundamentally incomplete knowledge of glucose and deoxyglucose metabolism in gliomas (e.g., the "lumped constant" for 2-deoxy-D-glucose is not yet established) and due to the limited size of our series it is not possible to designate a strict correspondence between absolute LCMRGlc and tumor grade at this time. Instead, the terms "hot" and "cold" are used, based on tumor isotopic activity relative to activity in remote cerebral cortex (representing "hot" activity) and white matter ("cold") in the same scan plane. The correspondence of "hot" lesions to higher grades of tumor and the converse correspondence of "cold" lesions to lower grades is evident from the table. The apparent exception to this correspondence in patient 12 may be explained by a good response to therapy at the time of the PECT scan which has been followed by a stable course for a period of 6 months. All other patients scanned post-operatively have shown progressive clinical deterioration with CT evidence of recurrent tumor in the 6 months following the PECT scan. In patient 13, scan "a" was done within one month of the end of chemotherapy and the repeat PECT scan 4 months later was associated with clinical deterioration and clear CT evidence of recurrent tumor.

An interesting finding in the pre-operative patients with hemispheric lesions (1,3,4,6,7) is a suppression of adjacent cortical LCMRGlc by as much as 50% relative to contralateral cortex, which does not seem to be explained simply by a volume effect of peritumoral edema.

In our laboratory, biochemical studies of glucose utilization in cultured human glioma cells have revealed values similar to those measured in tumors in vivo by  $^{18}\text{F}$ FDG-PECT. A validation of tumor LCMRGlC measured by  $^{18}\text{F}$ FDG-PECT is being obtained by direct correlation with simultaneously determined LCMRGlC using  $^{14}\text{C}$ FDG autoradiography in a monkey cerebral glioma model. Correlation of  $^{18}\text{F}$ FDG-PECT with  $^{14}\text{C}$ -FDG autoradiography in resected human cerebral glioma is contemplated.

TABLE I

Pt. No.	Glioma Histologic Grade (Kernohan)	PECT	CT	
			enhancement	calcification
1.	IV	"hot"	+	-
2.	IV	"hot"	+	-
3.	II	"cold"	-	+
4.	I	"cold"	-	+
5.	N.A.	"cold"	+	+
6.	N.A.	"cold"	-	+
7.	N.A.	"cold"	-	+
8.	"Oligo./malig. foci"	"hot"	+	-
8a.	"Oligo./malig. foci"	"hot"	+	-
9.	IV	"hot"	+	+
9a.	IV	"hot"	+	+
10.	III	"hot"	+	-
11.	IV	"hot"	+	-
12.	III	"cold"	+	+
13a.	IV	"cold"	+	-
13b.	IV	"hot"	+	-

Patients 1-7 were scanned prior to surgery, chemotherapy, or radiation therapy. Histologic grade in cases 1-4 was determined on surgical specimens within one month after the PECT scan. Patients 5-7 had histories of stable symptomatology for more than 2 years at the time of the PECT scan and have not yet gone to surgery.

Patients 8-13 were scanned following surgery, chemotherapy and radiation therapy. Histologic grade in these cases was determined on surgical specimens within 9 months prior to PECT scan in 8,11,12,13 and at 5 months after and 2 years prior to PECT scan in 9 and 10, respectively. Scans in patients 8 and 9 were repeated after a 6 month interlude.

At the time of PECT scan, all patients except 5 were treated with therapeutic doses of anticonvulsants and in addition patients 1,2,8,9,11, 12 and 13 (scan "b") were treated with oral steroids.

2) The protocol project " $^{18}\text{F}$ -2-Fluoro-2-deoxy-D-glucose (FDG) Positron Emission Computed Tomography (PECT) in Epilepsy" is also well under way.

Positron emission tomography (PET) was performed with  $^{18}\text{F}$ FDG in nine patients (ages 20-39) with normal CT and neurological exams who had



refractory complex partial seizures. All were studied with simultaneous EEG monitoring. Four patients had only unilateral epileptiform discharges, two had predominantly unilateral discharges, and three had diffuse or bilateral epileptiform abnormalities. Except for two patients with bilateral discharges, all had a hypometabolic lesion demonstrated by PET. PET was not affected by (1) the seizure frequency, (2) medication changes, (3) state of alertness, or (4) number of spike discharges. PET abnormalities were affected by the presence of simultaneous clinical seizures. In one patient, a seizure which occurred 90 minutes prior to FDG injection did not alter the hypometabolic area. In another patient, a seizure 18 minutes prior to injection produced an excessively hypometabolic area compared with an interictal scan. In a third patient, sequential scans showed progression from an ictal hypermetabolic to a postictal hypometabolic state. PET requires careful clinical and EEG correlation; the metabolic changes in complex partial seizure patients are stable only if a recent seizure has not occurred. Focal lesions may be demonstrated even if the abnormality on routine EEG is diffuse. For patients whose neurological and CT exams are normal, PET is especially significant, as it may allow noninvasive localization for surgical candidates.

3) Work has just started (one patient) on a third protocol "Positron Emission Tomography to Determine Regional Cerebral Metabolism at Rest and During Cognitive or Pharmacological Stimulation in Patients with Alzheimer's Disease, Huntington's Disease and Those at Risk for Huntington's Disease".

4) Two new protocols are in the approval/preparation stage, one dealing with PET in dyskinesia and the other dealing with FDG-PET in Parkinson's Disease.

5) The construction of the new, high resolution, NIH-built PECT-scanner (the Neuro-PET) is very advanced. Phantom testing should begin in the next few weeks and patient scanning in several months.

6) A great deal of activity has been spent to solve the problems connected with the choice, purchase, and location/installation planning for the cyclotron at the NIH.

Significance to Bio-Medical Research and the Program of the Institute: The FDG-PET studies represent a totally new approach to the understanding of the physiopathology of many neurological diseases. Undoubtedly the information acquired will be applied to the patients' management.

Proposed Course of Project: The clinical studies with FDG-PECT are under way. The collected observations are being analyzed and interpreted. Construction of the new high-resolution PECT-scanner, the Neuro-PET, is coming to its final stage.



Publications: Brooks, R.A., Sank, V.J., Friauf, W.S., Leighton, S.B., and Di Chiro, G.: Design considerations for positron emission tomography. IEEE Trans. Biomed. Eng. BME-28:158-177, 1981.

Di Chiro, G., DeLaPaz, R.L., Smith, B.H., Kornblith, P.L., Sokoloff, L., Brooks, R.A., Blasberg, R., Cummins, C., Kessler, R.M., Wolf, A.P., Fowler, J., London, W.T., and Sever, J.:  $^{18}\text{F}$ -2-fluoro-2-deoxyglucose positron emission tomography of human cerebral gliomas. In: Proceedings of the Tenth International Symposium on Cerebral Blood Flow and Metabolism, St. Louis, 1981, (in press.)

DeLaPaz, R., DiChiro, G., Smith, B.H., Kornblith, P.L., Quindlen, E.A., Sokoloff, L., Brooks, R.A., Kessler, R.M., Johnston, G.S., Manning, R.G., Flynn, R.M., Wolf, A.P., Fowler, J.S., Brill, A.B., Blasberg, R.G., London, W.T., Sever, J.L., Kufta, C.V., Rieth, K.G., Goble, J.C. and Cummins, C.:  $^{18}\text{F}$ -2-fluoro-2-deoxyglucose positron emission tomography of human cerebral gliomas. AANS Scientific Manuscripts, 29-30, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02010-09 SN																																				
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SUMMARY OF WORK (200 words or less - underline keywords) Extracellular and intracellular responses of the ganglion nodosum to stimulation of the vagus nerve were recorded. Based on the conduction velocity of these extra and intracellular responses, the ganglionic cells appeared to respond mainly to the impulses initiated from the C-fibers; and, occasionally also from the A-delta fibers of the vagus. Investigation of the electrical properties of the ganglionic cells (e.g., input resistance, capacitance, time constance, change in membrane potentials and discharge activities) in response to electric stimulation of the vagus and superficial peroneal nerves and to application of chemical agents in process. At present, no conclusive statements can be made.																																						

PROJECT DESCRIPTION:

I. Objectives:

- a. To investigate the neurophysiological mechanisms of pain sensation.
- b. To study the effect of conditioning somatosensory inputs upon the response of neurons which are activated by pain-fiber stimulation.
- c. To study the effect of change in neurotransmitters in relation to the response of neurons activated by pain-fibers stimulation.

II. METHODS EMPLOYED:

- a. Adult cats under light general anesthesia were used.
- b. The vagus nerve (right or left) and the ganglion nodosum were exposed.
- c. The vagus nerve was placed in a specially constructed stimulating-recording chamber while the ipsilateral superficial peroneal nerve was similarly exposed, stimulated, and recorded.
- d. The ganglion nodosum was penetrated by either a single or multibarrel micropipette electrode. The responses and changes in membrane properties of the ganglionic cells were recorded before and after nerve stimulation and application of pharmacological agents through iontophoretic injections.
- e. Similar investigations are to be carried out in the nerve cells of the nucleus tractus solitarius and the nucleus ventralis posterior medialis of the thalamus, and the sensory cortex.
- f. The pain fibers and the neurons with dendrites and axons involved will be studied by horseradish peroxidase and electron microscope.

III. MAJOR FINDINGS:

There are no conclusive statements that can be made at this time.

IV. SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE:

The present study will eventually provide a better understanding of the mechanisms of pain.

V. PROPOSED COURSE OF THE PROJECT:

To continue the present study and to reorganize the laboratory by requesting a full time and a dedicated technician to facilitate our research programs.

VI. PUBLICATIONS: None









ANNUAL REPORT

October 1, 1980 through September 30, 1981

Laboratory of Central Nervous System Studies

National Institute of Neurological and Communicative Disorders and Stroke

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PUBLICATIONS	41-LCNSS/IRP -- 57-LCNSS/IRP



October 1, 1980 through September 30, 1981

Laboratory of Central Nervous System Studies  
National Institute of Neurological and Communicative Disorders and Stroke

The Laboratory of Central Nervous System Studies comprises two major projects: (1) Neurobiology of Population Isolates--the Study of Child Growth and Development, Behavior and Learning, the Disease Patterns in Primitive Cultures; and (2) Chronic Central Nervous System Diseases Studies--Slow, Latent and Temperate Virus Infections. Both projects are an outgrowth of the Study of Child Growth and Disease Patterns in Primitive Cultures. It was this parent project that gave rise to the discovery of kuru, a hereditary subacute progressive degenerative disease of the central nervous system of the Fore people and their neighbors in the Eastern Highlands of Papua New Guinea, and led to the demonstration that kuru is caused by a serially transmissible virus which possesses unconventional biological and biochemical properties. This was the first demonstration that chronic degenerative disease in man could have virus etiology and directly stimulated the research that led to the discovery of several other slow virus infections of man. The successful transmission of kuru and the isolation of its virus provided the necessary techniques for the subsequent discovery of a viral etiology for some forms of presenile and senile dementias of man, particularly the Creutzfeldt-Jakob type (CJD), and it was this study that has led to the discovery that the agents causing these diseases form a group of transmissible virus-like agents new to the field of microbiology. These are the only known virus infections without examples of recovery and are unique in their total failure to evoke any immune response to the causative virus. Moreover, familial forms of CJD appear to be the first examples of virus disease of man with genetic (single gene) control of pathogenesis.

Additional foci of high incidence of disease of great general importance to all of medicine have been located and studies on all of these are underway. These include: (1) focus of high incidence of ALS and PD in West New Guinea among the Auyu and Jakai peoples; (2) focus of high incidence of motor neuron disease among Australian aborigines on Groote Island and adjacent Arnhemland; (3) focus of high incidence self-limiting epilepsy as a newly recognized form of cerebral cystercercosis in West New Guinea; (4) focus of high incidence premature aging in certain highland populations in New Guinea; (5) focus of unusually high incidence and early age of appearance of amyloid plaques and neurofibrillary tangles characteristic of neurological aging in certain isolated populations; (6) foci of very much delayed menarche and male and female puberty in isolated Melanesian populations; (7) foci of high incidence spinocerebellar ataxias of diverse types in very isolated highly inbred populations on la Reunion Island in the Indian Ocean; (8) foci of high incidence Huntington's disease in several isolated Amerindian (Venezuelan) and Melanesian (Papua and New Britain) populations; (9) focus of high incidence male pseudohermaphroditism in isolated Melanesian and Australian aborigines; (10) foci of high incidence of presenile dementias of a slow virus etiology in several population isolates; (11) focus of high incidence of familial parkinson's disease in the Agau Papuan population; (12) foci of extremely high incidence of goitrous cretinism with congenital CNS defects including deafness, mental and motor defects in New Guinea highland populations; (13) focus of congenital Still's disease on Satawal Island, Western Caroline Islands; (14) foci of abnormally high incidence of

chronic lung disease, the leading cause of death, and associated with an extraordinarily high incidence of bronchial asthma in childhood on Micronesian islands; and (15) foci of high incidence hyperuricacidemia including juvenile gout on Micronesian islands.

These studies have continued from their roots in the investigation of kuru, which has been detailed in the Monograph published in 1981: "Kuru: Letters and Field Notes from the Collection of D. Carleton Gajdusek", dealing with the first year of kuru investigation. The field journals (thirty-two volumes) and research cinema documents dealing with our work in isolated and primitive populations over the past twenty-five years are now being used extensively in the studies of child behavior and neuromuscular development, age and speed of puberty, age of menarche, and patterns of aging; different culturally determined patterns of learning, language acquisition, memory, cognition and symbolic representation; differing time, numerical and other quantitative senses and unusual forms of psychosexual development; development and patterns and psychiatric breakdown, juvenile suicide, violence, outbursts of unusual mass hysterias, use of drugs, and other fad-like stereotype behavior patterns in diverse, isolated, primitive social and cultural settings.

Other studies of man in isolated and primitive groups as opportunistic investigations of importance to medicine on a worldwide basis were highlighted in the Silliman Lectures in April of 1981 at Yale University (DCG), which summarized the results of such research:

#### ISOLATION AND THE CONDITION OF MAN: NATURAL EXPERIMENTS IN HUMAN DIVERSITY

1. High Incidence Foci of Chronic Neurological Diseases in Isolated Groups
2. New Viruses Causing Old Diseases: Kuru and CJD
3. Unique and Exotic CNS Patterning in Infancy and Childhood as a Consequence of Isolation
4. Unusual Patterns of Genetic and Infectious Diseases as a Consequence of Isolation

These lectures are being prepared into a monograph to be published by Yale University Press.

#### THE NEW GROUP OF MICROORGANISMS CAUSING THE SSVEs

Following the convening of a series of international workshops on the "Subacute Spongiform Virus Encephalopathies and the Structure of the Unconventional Viruses Which Cause Them" held in the latter part of 1978, the staff of LCNSS participated in an international symposium on "Slow Virus" sponsored by NIAID and held at the Rocky Mountain Laboratory, Hamilton, Montana. Eleven papers were presented and have been published (Academic Press); they covered the origin of studies on slow infections in humans, the worldwide epidemiology of these diseases, the pathogenesis and molecular biology of the viruses, the biological, physical and chemical properties of the viruses including the evidence for strain variations and their unusual resistance to gamma and ultraviolet radiation.

The most challenging outcome of the discovery that some chronic progressive non-inflammatory CNS diseases (sporadic, as most cases of Creutzfeldt-Jakob disease (CJD); epidemic, as kuru; or familial, as familial CJD and kuru) are



"slow infections" caused by viruses with incubation periods measured in years or decades, has been the realization that the etiologic agents of these infections are a new kind of microorganisms. The absence of antigenicity and their unusual resistance to ultraviolet and ionizing radiation, to formaldehyde and other disinfectants such as  $\beta$ -propiolactone, ethylene oxide, and to heat place them in a group unique among viruses. Their ability to produce fatal CNS disease without eliciting inflammatory responses, the failure of the course of disease or incubation period to be influenced by immunosuppression, and failure to demonstrate any antigenicity in high titer infective virus preparations, or to find any evidence of humoral or delayed hypersensitivity reactions in the diseases, as well as an absence of response to interferon, stimulation of interferon, or interference with interferon production, and absence of interference with known viruses, form the series of atypical biological properties which likewise differentiate these agents from any other group of microorganisms. On the other hand, classical virus properties, such as adaptation to new hosts, broadening of host range and reduction of incubation period, dependence of pathogenic effect on the genetic breed of the host, the presence of strains of differing virulence in wild stock viruses selected by limiting dilution, and the interference of fast-growing by slow-growing strains of scrapie, are all indicative of a complex host-virus genetic interaction characteristic of more classical viruses. Attempts to delineate the chemical nature of the replicating agents, especially to determine whether they are replicated from introduced genetic information or by the induction, derepression or activation of pre-existing genetic information in the host, are the major thrusts of current investigation.

The elucidation of the structure and molecular configuration of the infectious agent of scrapie, CJD, and kuru remains the first goal of this laboratory. For two decades this frustrating problem has been a challenge to molecular biologists, biochemists, and virologists in many laboratories.

In the past year we have made advanced in our attempts to characterize the scrapie agent:

A. Cesium chloride fractionation of the infectivity. The general trend of the infectivity distribution in the first sedimentation to equilibrium from homogeneity of the mouse scrapie agent from a mouse brain homogenate has been determined. The infectivity is banding in a broad peak centered around density 1.24. The broadness of the peak indicates a considerable heterogeneity in density. Due to the steepness of the gradient we have achieved a marked separation from other components assayed, i.e. RNA, DNA, protein and lipid. The preliminary infectivity data also indicate that the cesium chloride gradient has concentrated the infectivity relative to a sample stored in cesium chloride and not banded. Purification of 500x with respect to total brain DNA has been achieved.

Individual or combined fractions from these gradients have been assayed analytically for scrapie specific DNA, RNA and proteins by gel electrophoresis but as yet without detecting a new species of macromolecule. The highly complex protein patterns are virtually identical in normal and affected brain except for several protein deficiencies in the affected animal.

Study of the behavior of scrapie infectivity with exposure to high energies of sonication with rise in infectivity titer and fall even on frozen storage

thereafter, indicate "sticky" clumping of the infectious units. Theoretical reinterpretation of much of the scrapie inactivation data in the light of the newly proved association or aggregation of infectious units indicates that even the aberrant behavior to UV and ionizing radiation may still be consistent with a larger virus than we previously suspected.

B. Adaptation and development of the hamster 263-K strain of scrapie with high virus yields, shorter doubling time and shorter incubation periods than in mouse scrapie. Scrapie-infected hamster (strain 263-K) is a more suitable source of virus for purification studies. It is associated with a short incubation period and high initial titer of infectivity. The disease can be detected behaviorally only 55 days after a high titer passage, compared with a minimum of 180 days in the mouse system. Several titrations of hamster 263-K brain homogenates have consistently shown initial brain titers of  $2-5 \times 10^{10}$  infectious units/gram of brain, over 100 times the titers obtained from mice. In a detailed analysis for biochemical studies and titration purposes, the hamster system is at least two times and, for some purposes, over 500 times more efficient with respect to titration time and required animal space than is the mouse system. In terms of macromolecular distributions the hamster brain has fractionated much the same as the mouse brain. There is also a pronounced dependency of incubation time in the hamster on the dose of the agent, and this feature of the disease can be exploited to give an early indication of the distribution of the agent in fractionations, if not a quantitative assessment of infectivity.

C. The possibility of obtaining infectious nucleic acids from extracted brain tissue. In order to enhance the potential infectivity of any naked nucleic acid recovered by our procedure we coupled the infectious assay with a transfection procedure which we had shown to be effective for herpes simplex virus, OX-174. The experimental approach was to fractionate infected mouse brain homogenate following a heat inactivation step (80°C for 30 minutes) designed to inactivate any enzymes that might interfere with the recovery of infectious material. Following heat inactivation the homogenate was digested with Protease K, then extracted with phenol in the presence of 1% sodium dodecyl sulphate (SDS). The resulting three fractions (aqueous, phenol and heavy interphase) were further extracted under conditions designed to preserve the molecular nature of the material finding its way to that fraction. The aqueous phase was further extracted with organic solvents and alcohol precipitated. The phenol phase was buffer extracted to recover any material and the interphase was buffer extracted to remove the phenol. The resulting fractions were assayed for infectivity in NIH Swiss Webster mice. The results of this experiment clearly indicated that there was no infectivity associated with the nucleic acid fraction. The conditions used in these experiments yielded infectious HSV-1 DNA from infected cells but provided no scrapie infectivity. The heat and Protease K treatment had no effect on the infectious titer, however the subsequent steps destroyed virtually all of the infectivity. The only possible infectivity should have been in the highest concentrations of the buffer extracted interphase from the phenol extraction; the presence of infectivity in this fraction has not been confirmed by pathology. These results suggested to us that the viroid model, at least in its simplest forms, is not valid for the unconventional agents. Further studies on the scrapie system have focused on our impression that an essential, very hydrophobic protein is intimately associated with the scrapie agent and that new procedures are necessary for its isolation.

D. Attempt to detect double-stranded scrapie-specific DNA by molecular hybridization. More recent studies reported in the literature indicate that at least a small percentage of the scrapie population has a DNA component of low molecular weight that is DNAase sensitive which is eluted at 0.48M phosphate buffer from hydroxyapatite columns. This would suggest that the DNA molecule could be double stranded. During this year we tried to detect double-stranded scrapie-specific DNA by molecular hybridization experiments since analysis of the kinetics of DNA reassociation has proven to be a very sensitive means of detecting the presence of specific DNA sequences in mammalian genome. As a probe we used the DNA extracted from concentrated enriched scrapie labeled with  $I^{125}$  and annealed to total DNA extracted from infected and uninfected brains of the same and different species. No difference was observed between the extent of reassociation of the probe with DNA of scrapie or normal animals. Our levels of detection indicate that if the scrapie agent were a double-stranded DNA molecule its presence in infected brain tissue is below the level of 50 molecules of DNA per infective unit. We have sought also to repeat the work of others claiming to have isolated a scrapie-specific DNA. However, our attempts to reproduce this much discussed procedure are disappointing with less than a 1% recovery of infectivity in the high speed supernatant as opposed to the 10-90% indicated by Marsh and Malone. When this high speed cell-free virus was placed on a 2.5% polyarylamide-0.5% agarose gel (9.5x0.6cm tube) at 6 mA of voltage for 2 hours, all of the infectious virus entered the gel and was recovered ( $4.8 \times 10^6$ ). Enzyme treatment of these infectious units was not interpretable due to the total inactivation of the virus at 37°C after 3 hours. These studies are being continued.

E. Comparison of neurotransmitter concentrations in brains of scrapie-affected and normal mice and hamsters in the hope of identifying a particular neuronal system as the target for the infection in the brain. Comparing late scrapie mice with same age controls we have observed normal levels of catecholamines and most amino acids, but a two-fold increase in GABA levels and a nearly 100-fold decrease in 5-hydroxytryptamine (5-HT) levels. This finding prompted us to look at 5-HT levels in the blood. In the case of late hamster scrapie we observe a somewhat variable but significant decrease in blood serotonin of almost two-fold. At present these findings are being vigorously pursued: (1) to discover the time course of these changes and correlate them with behavioral changes and histopathology; (2) to narrow down by behavioral neuropharmacology, and brain microassay of neurotransmitters and enzymes the specific lesion(s) involved; (3) to identify other non-CNS indicators of these changes which may be of clinical use; and (4) to test the efficacy of 5-HT analogs as a therapy.

Our studies on the therapeutic benefits of the serotonin agonist, quipazine maleate, and the serotonin precursor, L-5-hydroxytryptophan methyl ester, on scrapie infectious hamsters have shown that both drugs effect small but statistically significant improvements on ataxia and action jerks within a rather narrow dose range. At higher doses we observed a dramatic hypersensitivity in the scrapie infected animals to the toxic effects of both drugs. This hypersensitivity syndrome is an intensively studied phenomenon in the rat and has been shown to originate in that system from neuropharmacological destruction of serotonergic nerve terminals. The hypersensitivity that we have observed in the hamster is even more than that which can be induced by neurotoxic agents in the rat. Thus we may support that the scrapie infection in the hamster results in the destruction or degeneration of the axon terminals of



the serotonergic nerves. This is the first example of a serotonin hypersensitivity arising as the consequence of a natural disease state.

In our studies of the biochemical levels of serotonin in the brains and blood of scrapie infected hamsters and mice we have observed the following: (1) a highly significant 2.5-fold decrease in the blood serotonin levels in scrapie infected hamsters but no similar change in mice; (2) a highly significant 20% reduction in mouse brain serotonin levels but no similar change in hamsters. This change in mouse brain concentrations is seen only in the late clinical stage of disease; and, (3) a much larger 10-fold decrease in mouse brain serotonin levels after frozen storage for a prolonged period. Our observation of a 2.5-fold decrease in blood serotonin levels in scrapie infected hamsters is the first major change in blood chemistry noted in the subacute spongiform virus encephalopathies.

F. In a continuing effort both to characterize scrapie virus and find ways to inactivate and/or stabilize it we have performed the following inactivation experiments: (1) sensitivity of scrapie to shear forces; (2) sensitivity of scrapie to osmotic shock; (3) sensitivity of scrapie to exhaustive protease treatment; and (4) sensitivity of scrapie to chlorine dioxide. Results of these studies show: (1) overall scrapie infectivity in brain homogenates can be increased at least 17-fold by exhaustive sonication immediately prior to titration. This quantifies to some extent the level of aggregation of scrapie virus in the usual preparations. We have extended these studies to determine whether or not the high intensities of sonic radiation used in these experiments are inactivating infectivity as well as dissociating aggregates as well as investigating the kinetics of reaggregation. (2) Much of the infectivity loss often associated with exposure to high ionic strength buffers is apparently due to enhanced aggregation under these conditions. (3) If scrapie is inactivated at all by powerful proteases this occurs at a much slower rate than for brain homogenate proteins in general. (4) A kinetic analysis of the inactivation of scrapie infectivity by sodium hypochlorite and chlorine dioxide, show both chemicals to be equally effective inactivating 99.9% of the population in the first few minutes of exposure.

A critical analysis of ionizing radiation data and electrophoresis of scrapie has been undertaken during this past year. The conventional wisdom is that the infectious agents of the subacute spongiform virus encephalopathies (SSVE) are very small, probably even subviral in size. A favorite hypothesis is that they may represent examples of animal viroids. This expectation is based upon the well established resistance of the SSVE to inactivation by ionizing radiation and, more recently, the observation that scrapie infectivity will comigrate with a viroid marker in some electrophoretic gel system. Dr. Rohwer in our laboratory has now offered intriguing alternative interpretations for both of these findings. He has shown that if the SSVE are highly aggregated, as his sonication data indicate (see above), then the traditional first order analysis of the ionizing radiation data is inappropriate. If aggregation is taken into account in the analysis of the inactivation kinetics, the actual size of the scrapie agent must be much larger than that deduced previously from a first order inactivation constant and, in fact, is consistent with the molecular weight of ordinary viruses. He has also shown that, in the electrophoretic systems used to characterize the mobility of the scrapie agent, viruses fractionate on the basis of their charges whereas nucleic acids fractionate on the basis of their molecular weights. In these same systems simple

bacteriophages comigrate with much smaller nucleic markers and in fact the two species cannot be used to calibrate one another and separations such as these cannot distinguish viruses and viroids.

#### FAILURE OF SCRAPIE INFECTION TO INDUCE AN IMMUNE RESPONSE AND LACK OF ANTIGENICITY OF SCRAPIE VIRUS IN HIGH INFECTIVITY TITER

A. During the period covered by this report major efforts have been made to study the interaction of scrapie with the immune system of infected animals. These studies have been done in three parts. First, the search for a new antigenic component on the surface of spleen cells at various times following infection. Second, a systematic examination of the interaction of scrapie with a C3H/HeJ mouse line reported to be unique. Thirdly, the identification and culture of the infectious cell population in the mouse spleen.

The search for a new antigenic component of the surface of spleen cells was based on the possibility that a new cell surface component would not be detected by the humoral immune response but would be detected by the cellular immune system. To examine this possibility, mixed lymphocyte cultures were utilized using two inbred strains of mice, Balb/C and C57BL/6. Two large groups of animals were studied with cultures at weekly intervals over the early and late stages of infection. In every case controls inoculated with normal mouse brain were included on a 1:1 ratio. Data during the early post infection period included spleen weights to check for the enlargement reported by others. Throughout this study the results were uniformly negative with respect to both the splenomegaly and to the presence of any new cell surface component. Several cultural combinations were included to examine the scrapie-infected cells as both target cells and responder cells. It seems clear from this work that: (1) there is no new cell surface component on scrapie-infected spleen cells that can be detected in mixed lymphocyte culture; (2) scrapie-infected spleen cells retain the capacity to respond to the mitogens Con A and LPS as well as respond to a heterologous H-2 determinant in mixed lymphocyte culture. These responses are identical in magnitude to those animals inoculated with normal mouse brain; (3) there is no detectable splenomegaly in scrapie infected mice within the first three months of infection and there is no splenomegaly throughout most infections.

Extensive studies with the C3H/HeJ strain of mouse have not confirmed the published report of other investigators that this strain of mouse, when infected with scrapie, loses its ability to mount a mitogenic response to the endotoxic protein component of *E. coli* LPS. This animal is genetically unable to respond to the Lipid A moiety. These studies were carried out at weekly intervals from weeks 2 through 7, since previous reports indicated the peak depression to occur at week 4. It has been reported that a marked spleen enlargement occurred, a finding also not confirmed in this work. There are only two possible explanations for the lack of agreement--one is a difference between the Chandler and C506 strains of scrapie, or that other investigators had a contaminating virus in their inocula. The plan for the future is to attempt to determine which of these is the explanation and to attempt to clarify completely if there is or is not a measurable change in the immune response of C3H/HeJ mice with scrapie.



The results of the spleen cell sub-population studies have been completed. It is clear that strain C506 gives extremely low spleen titers and that only a very small number (less than 1 in  $10^5$ ) spleen cells are infectious, whatever sub-population they are in. Extensive studies on splenic macrophages in culture have been disappointing from the point of view of continued infectivity.

We have also explored the ability of scrapie to grow in vitro in well-established, 'T', 'B' and macrophage cell lines of murine origin. Two questions are being investigated: (1) does the cell have a receptor for scrapie on its cell surface?; and (2) if it does not have a receptor (assuming that scrapie agent is the free nucleic acid bound to lipid membranes), do other methods have to be used to get the agent in the cell so that it could replicate? Inactivated Sendai virus and lysolecithin were used as membrane-fusing agents; DEAE-Dextran, which alters the permeability of the membrane and is used for assay infectivity of other viral nucleic acids in cell culture, was also used. Cell culture harvests from these experiments have been titrated in mice for infectivity and the results from these experiments will help us answer the two questions. Since most of the murine cell lines used in the study have endogenous C-type viruses, it will also be interesting to see if these viruses act as helper viruses for the growth of scrapie. Attempts to grow scrapie in mosquito cells: Aedes albopictus mosquito-cell lines have been used to grow several groups of arboviruses. In such cells these viruses grow at 22°C without producing cytopathic effect, and infected cells become chronically infected by the virus. Virus is released from these chronically infected cells into the medium. We have inoculated these cells with the scrapie agent, and cell lysates at different passage levels have been inoculated into mice for the assay of infectivity. Results were discouraging since unlike some members of the togaviruses, scrapie infectivity was not recovered from inoculated insect cell lines. An SV-40 transformed cell line that contained scrapie virus at the 12th passage level was serially passaged to higher levels; none of 50 pooled and cloned cultures was infectious for mice at the 30th passage level or higher. The scrapie-infected SMB line of Clarke and Haig was imported from England; five lots of this line have been prepared and aliquots stored; mutants of the cells are being prepared. A line of cells was derived from the brain of a hamster infected with the 263-K strain of scrapie; this line is also under study.

B. Since conventional immunological techniques have thus far failed to elicit an antigen-antibody reaction in kuru, Creutzfeldt-Jakob disease or scrapie, we have been attempting to produce specific antibody to scrapie by the hybridoma technique of Kohler and Milstein since it has been shown that cells from a mouse myeloma could be fused with splenic cells from mice stimulated with an antigen, and such fused cell clones produce specific antibody which is monoclonal for individual antigenic determinants. Such a technique facilitates antigenic analysis of complex antigens. In our studies spleen cells from mice immunized with scrapie infected mouse or hamster brain scrapie specific antibody has not yet been obtained; however, 30 monoclonal antibodies were derived which are reactive to antigens in hamster or mouse nervous system tissues. Of the 30 clones analyzed, specificity included clones reacting with grey matter of mouse and hamster brain, one clone reacting with axons in animal brain, several clones reacting with cytoskeletal proteins (intermediate and micro-filaments) and 19 clones which produced antibody reactions with both neural and non-neural tissue components.

C. We also measured the general immunocompetence of splenic lymphocytes in an attempt to detect alterations of the immune system of scrapie affected animals. In general splenic activation by Concanavalin A, phytohemagglutinin and lipopolysaccharide of control and scrapie inoculated mice were compared. Mitogen-induced responses of splenocytes from infected and control cultures were not significantly different. The PHA response of scrapie-infected mouse spleen cells was slightly depressed over a period of 29 to 56 days post-inoculation. Additional efforts to induce scrapie specific antibody are underway and indeed the use of several different preparations of high-titering scrapie infected hamster brain that has been subjected to (a) chemical tissue membrane modifiers, (b) purified by density gradient banding, and (c) tied up with haptens. Such mitogens are being assayed in animals rendered immunotolerant to uninfected hamster brain.

As a control for the scrapie studies, somatic cell hybridization to produce monoclonal antibody against a major glycoprotein (P<sub>0</sub> 30,000 MW) associated with human peripheral nervous system myelin was carried out. Thus far we have produced two clones both of which react with peripheral nerve myelin; only one produces antibody specifically reactive with the P<sub>0</sub> low molecular weight glycoprotein.

D. Since the demonstration of cell-fusing activity in the majority of brain extracts of scrapie mice and CJD patients (see ANNUAL REPORT: October 1, 1977 through September 30, 1978) additional studies have been carried out using two different techniques. One involved the formation of multinucleated cells and the other the formation of somatic hybrid cells. Heterokaryons were measured at 18 hours and hybrid cells after an average of 25 days. The studies employed three scrapie cases, 32 cases of transmitted CJD, two cases of untransmitted CJD, 26 cases of other neurological diseases, three transmitted cases of other than CJD and 17 patients without neurological disease. The results show a significantly higher proportion of CJD brains (61%) was positive than other neurological diseases (31.4%) or the control group (6%). Thus our earlier observations have been clearly confirmed and although the assay does not separate CJD from other neurological diseases to warrant its use as a specific diagnostic test we hope that such discrimination can be improved to the extent that the detection of cell-fusing activity might be possible utilizing serum, urine and CSF from patients and their family members as a biological marker of this disease. We shall continue to study the phenomena of cell fusing activity in an effort to elucidate the mechanism in CJD and other neurologic diseases as well as the application of this technique as a rapid means of more quickly measuring infectivity in experimentally derived fractions from purification procedures employed for scrapie and CJD.

Recently study of the appearance of this cell fusing activity in brain of hamsters infected with scrapie has shown peak fusing activity attained early in incubation (4 weeks) instead of during clinical disease (8 to 9 weeks). This may indicate the desirability of studying hamster brain early in the incubation period for possible biochemical markers of scrapie virus or scrapie activity.

E. Resistance to high concentration of formaldehyde, to heat up to 85°C, and to ultraviolet radiation at 254 nm, and an ultraviolet sensitivity at 237 nm greater than at 254 nm have been found for kuru and CJD viruses as for scrapie. These very unusual physical properties greatly emphasize our current contention that the viruses of the human diseases are closely related to the scrapie virus.

Similarly, the two human agents have been shown to have the same enormous resistance to ionizing radiation (gamma rays from Cobalt  $CO_{60}$  as is found for scrapie virus. The most direct inference from this enormous resistance is an effective size of under 100,000 daltons molecular weight. Although many possible explanations, including atypical fine structure for a nucleotide configuration and unusually efficient nucleic acid repair mechanisms have been suggested to account for such anomalous properties, the simplest explanations namely, that in fact the agents are of such small size, may be true; or, the new data of extensive "sticky" clumping or aggregation of infectious units may account for much of the anomalous behavior.

## REVISION OF SURGERY AND AUTOPSY ROOM TECHNIQUES FOR DEALING WITH DEMENTIA PATIENTS

A. Precautions for handling CJD patients in hospitals and in operating and autopsy rooms and laboratories. The discovery that the worldwide-distributed Creutzfeldt-Jakob disease is caused by a serially transmissible, self-replicating agent that passes through bacteria-, protozoan- and fungus-retaining membrane filters, the demonstration that the virus is widely distributed in non-CNS tissues and fluids of affected patients and possesses great resistance to usual antiseptics, has also resulted in a growing concern among medical and paramedical nursing and laboratory personnel, particularly neurologists, neurosurgeons, pathologists, and anesthesiologists, about the potential hazards involved in caring for patients with presenile dementias and handling their tissues. Concern comes largely from recent reports documenting transmission of Creutzfeldt-Jakob disease by corneal transplant, the accidental inoculation of two patients in neurosurgery with CJD-contaminated electrodes used in stereotactic electroencephalographic recording and stimulation, the suspicion that a neurosurgeon and two general practitioners may have contracted CJD from patients and the characteristic greatly over-represented among patients with CJD of a history of brain or eye surgery in the previous two years before onset of clinical disease. These concerns have further been heightened by the recent transmission of CJD to a chimpanzee by implantation of the same silver electrodes that had caused disease in the two human patients after more than two years storage in formaldehyde vapors used for sterilization. In response to these concerns we have published precautions for conducting biopsies and autopsies and have more recently, presented a summary on the current knowledge of the pathogenicity and communicability of CJD and related subacute spongiform virus encephalopathies of man and animals which are caused by similar unconventional viruses. We have also made recommendations on the rational precautions that should be taken in caring for these patients and in handling their tissues and helped establish guidelines for safe handling of the SSVE viruses in laboratories.

B. Studies on the inactivation of the SSVE viruses. During the last year, inactivation studies were made with disinfectants using mouse scrapie agent. Mouse scrapie, kuru, and CJD agents seem to have similar properties. Disinfectants used were clorox, organic iodine (Wescodyne), potassium permanganate, hydrogen peroxide, and Zepharin. Since ethylene oxide gas is commonly used in hospitals, ethylene oxide was also used. The data showed that after chlorox, a 1:250 dilution of  $KMNO_4$  was the most effective disinfectant, followed by Wescodyne and ethylene oxide, which reduced infectivity by 99



percent. Under the experimental conditions used in the study hydrogen peroxide did not affect the titer of the scrapie agent at concentrations used in the hospital environment. Residual toxicity of Zepharin for mice was high. Further studies are in progress on the CJD agent, with ethylene oxide autoclaving used for sterilization in the hospital setting. Finally, chlorine dioxide has been examined in parallel with potassium permanganate for inactivation activity against a guinea pig-adapted strain of CJD virus; and chlorine dioxide, sodium hypochlorite, potassium permanganate, hydrogen peroxide, and lysol® have been tested for activity against a hamster-adapted strain of scrapie. Time-dose experiments are on titration at this time, and should be completed within the year. Depending upon the results further recommendations will be made to the medical community. However, it is already apparent that some scrapie virus infectivity remains in hamster brain tissue of high titer after autoclaving and after ethylene oxide sterilization and that chlorox remains the most effective disinfectant.

## NATURAL HISTORY OF TRANSMISSIBLE VIRUS DEMENTIA The Search for the Source of Infection in Man

In an effort to determine the method of spread of CJD virus in man, we have recently completed a comprehensive worldwide epidemiologic survey of CJD. It is shown that in the United States the average annual mortality is at least 0.26 deaths per million population. Temporal-spatial clustering of cases was not found in the United States, but reports from other countries indicate that this occurs. Fifteen percent of the cases were of the familial type, suggesting a genetic susceptibility to infection. In this survey, some evidence was found that previous surgery or pre-existing neurologic disease may be associated with an increased risk of developing CJD.

A systematic investigation of all cases of CJD dying in France during the decade 1968-1977 was completed last year and updated through 1980 this year in collaboration with Dr. Francoise Cathala and members of the French Neurological Society, with a view towards clinical definition of a large and unselected case series, and to obtain some clue as to the natural mode of disease transmission. One hundred and seventy cases were discovered, of which 124, confirmed by autopsy or biopsy, were the subject of multifactor statistical analysis. The disease forms a clinical spectrum from nearly acute encephalitic type illness with a few weeks' rapid rapid progression and death, to lingering illness of years' duration, impossible to diagnose in the absence of neuropathological verification. Types of clinical onsets, range of symptoms during the course of illness, and symptom combinations with the highest frequencies were analyzed in detail. In addition, epidemiological data on all 170 cases were examined for the possibility of iatrogenic or case-contact types of human-to-human transmission. Apart from the approximately 10% of familial cases, no contact could be established between any two patients in France during a 10-year period, no iatrogenic transmission was discovered, no case occurred in any member of the medical profession, and those cases in paramedical professions did not occur at a higher rate than in the general population. Close examination of familial cases established that even in such families, personal contact between two subsequently affected members does not always occur, suggesting ever more strongly the participation of predominantly genetic factors in the familial type of CJD. Our epidemiological studies have already indicated that an annual incidence of nearly one case per million can be expected when newly occurring

cases are actively searched out. The frequency of the disease continued to be highest in the densely populated center of Paris, raising further speculation about human-to-human modes of natural transmission. On the other hand, study of exceptionally isolated cases, which could simplify examination of the number of possible routes of acquiring the disease, still has not yielded any clues to this problem. A full-scale study of any possible association of CJD and scrapie in sheep is also under way.

A detailed analysis of the clinical features of the first 100 transmissible cases of CJD has been performed, and the results compared to the clinical features of a similar number of cases of Alzheimer's disease. There is a considerable overlap in the clinical spectrum of both diseases, and a group of patients with Alzheimer's disease with myoclonus has been delineated for further clinical and pathological evaluation. In addition, the clinical syndrome of "amyotrophic" CJD and a group of cases of "untransmissible" CJD are being studied.

Other clinical features of CJD which may be related to different strains of the virus are being examined. A manuscript is in preparation describing a small number of cases of CJD with the clinical features of progressive supranuclear palsy. The differences between the acute and chronic forms of CJD have already led to the discovery of a virus strain from a Japanese case that takes readily in non-primates and causes both gray and white matter spongiform lesions. The possibility that the virus also causes previously unrecognized childhood encephalopathies is also being investigated.

In a continuing investigation on the possible modes of natural transmission of the CJD virus, we are intensively evaluating the familial occurrence of the disease. To date, we have identified 37 families with a total of 155 affected members. CJD occurs in a pattern suggesting autosomal dominant transmission. Compared with the sporadic form of CJD, in familial CJD the age at death is slightly earlier and there is a female preponderance. The clinical and pathological features are otherwise indistinguishable. No maternal effect was found. There was some evidence for anticipation. An analysis of temporal and spatial separations between affected family members suggests that if contact transmission were occurring, incubation periods up to four decades might be expected. However, the available data do not yet allow us to distinguish between a genetic susceptibility to infection or some form of vertical transmission. Studies are in progress determining genetic markers, such as the HLA type, of both sporadic and familial CJD, which might give us an indication of the genetic component of susceptibility to infection.

#### NEUROPATHOLOGICAL SURVEILLANCE OF CJD AND KURU

A major part of our experimental studies on CJD include the routine screening of the brains of all animals dying after inoculation with various chronic neurologic diseases, since it is now known that in the case of the squirrel monkey at least, approximately 15% of the animals die without showing clinical signs of neurological disease. The topography of the spongiform change has recently been analyzed in more than 200 squirrel monkey brains, where the results indicate that considerable variation in the severity and distribution of the lesions occur. The differences between CJD, kuru and scrapie are being examined in both primate and non-primate hosts. The unusual white matter change produced by a Japanese strain of CJD in mice is being examined.



A re-evaluation of the spongiform change in human kuru is being performed to see if the same general features as seen in human CJD also occur. The peculiar amyloid plaques that occur in 60% of kuru patients and approximately 10% of CJD patients is being investigated both structurally and at a biochemical level. The occurrence of these amyloid plaques in a virus-induced encephalopathy has great relevance to the etiology of the plaque of Alzheimer's disease.

#### SCRAPIE AND CJD VIRUS ALTERATIONS IN INTER-SPECIES PASSAGE

With our demonstration of the transmissibility of scrapie disease from American sheep and English goats to several species of non-human primates, manifested by a disease in the experimental monkey that is indistinguishable from the transmissible virus dementia originating from man, we are confronted with the urgent question of the possible relationship between scrapie of sheep and the spongiform encephalopathies of man. The scrapie virus is capable of infecting all species of monkeys tested. However, the Compton (English goat) strain after passage through non-human primates no longer induces disease when inoculated back into sheep or goats. Of tremendous importance has been the discovery that although these same strains of non-human primate-adapted scrapie virus did not induce clinical disease in mice during the more than two years they were observed, such mice did in fact have neuropathological lesions of spongiform encephalopathy in their brains and sub-inoculation of this material did induce disease in other mice. A similar observation has now been made on CJD in mice wherein transmission occurred on primary passage of human brain but on the first mouse to mouse passage animals remained asymptomatic for over 2-1/2 years yet when killed histopathological evidence of spongiform encephalopathy was observed in their brains. Thus, we have evidence that infected animals can remain asymptomatic and that in these animals the incubation period before onset of clinical disease may exceed the life span of the host.

The same exceptionally long incubation periods are evidenced in those few cases of kuru that have occurred in the Fore of Papua New Guinea during the past five or six years; new cases occur only in patients over 20 years of age.

#### PATHOGENESIS OF CJD IN MICE

The biological properties of scrapie appear to be altered after passage through the primate host--behavior, not unlike classical viruses; such altered biological properties may account for the failure of CJD and kuru viruses to induce disease in mice routinely. We have experienced difficulty in adapting the virus of CJD to mice and guinea pigs, but in recent experiments some passage lines of CJD have caused spongiform encephalopathy in both guinea pigs and mice, and we have recently completed studies on the pathogenesis of the Japanese strain of the virus in Balb-C mice. The findings were strikingly similar to the pathogenesis of scrapie in the mouse with a few notable exceptions. Initially, characteristic spongiform degeneration of the brain was first noted pre-clinically at 9 weeks following inoculation. Clinical signs did not become apparent until 16 weeks with the geometric mean incubation period being 112 days. Infectivity assays of various tissues of inoculated mice resulted in recovery of virus from brain and spleen as early as one week after inoculation. Furthermore, the average incubation period of mice inoculated with spleen was markedly less than that of mice injected with brain material from the second through the sixteenth weeks of incubation indicating that the concentration of virus is higher in the spleen than in the brain during the asymptomatic period.

Lesser amounts of virus were detected in thymus, lung, and kidney. In the kidney the virus appeared late in the pre-clinical period and the incubation period for recipient mice were prolonged. Virus was not detected in the liver in contrast to its presence in this tissue in human patients. Viruria was not demonstrable. However, we did confirm the presence of a viremia in CJD infected animals beginning during the sixth week after inoculation. Concentration of virus in the blood at the 14th and 18th weeks were estimated to be appreciable since the incubation periods in recipient mice ranged from 4 to 5 months. The clinical disease was confirmed histologically.

#### ORAL TRANSMISSION OF KURU AND CJD

We have now proven the transmissibility of the spongiform viruses by the oral route through feeding of virus-infected whole tissues. Two of two squirrel monkeys fed CJD-infected chimpanzee tissues and two of two squirrel monkeys fed scrapie infected whole tissues developed clinical disease and had typical pathological lesions of the spongiform encephalopathy in their brains. One of two monkeys fed kuru-infected chimpanzee tissues developed spongiform encephalopathy. The asymptomatic incubation period in the one monkey exposed to kuru was 36 months; that in the two monkeys exposed to CJD virus was 23 and 27 months, respectively; and that in the two monkeys exposed to scrapie virus was 25 and 32 months, respectively. The one additional animal similarly exposed to kuru has remained asymptomatic during the 45 months it has been under observation.

#### ANTI-NEUROFILAMENT ANTIBODY

The discovery of an heterogeneous autoantibody in the sera of kuru and Creutzfeldt-Jakob disease patients to neurofilament protein (Sotelo, Gibbs, and Gajdusek, SCIENCE 210:4466(October 10), 190-193, 1980) using mature neurons of murine origin in culture as antigens (Sotelo, Gibbs, Gajdusek, Toh, and Wurth, PNAS USA 77: 653-657, 1980), has initiated a series of in-depth studies to characterize the autoantibody and to determine whether or not it in any way shows specificity to the viruses causing the subacute spongiform encephalopathies. To date this does not on the surface appear to be the case since this autoantibody has been found in lower frequency in the sera of patients with other human neurological diseases. However, the possibility that our "unconventional viruses" utilize a host cytoskeletal protein in their structure as do some other viruses demands that this "non-specificity" be not too glibly dismissed. Already it is evident that its presence is not diagnostic of the subacute spongiform virus encephalopathies and its presence in high titer in the sera of Guamanian patients with amyotrophic lateral sclerosis and parkinsonism-dementia, patients with Alzheimer's disease, and other neurological diseases warrants this conclusion. However, the detection of this heterogeneous autoantibody has led to the particularly intriguing observation that it is remarkably specific for a small filament only in the axon of the cell unlike that of experimentally prepared antisera to neurofilament protein which reacts with filaments in both the axon and the dendritic processes of neurons. Finally, although unencumbered neurons of murine embryos in our *in vitro* test provide the best method for the detection and study of this immune reaction, its detection in mass screening has been much facilitated by the use of the indirect fluorescent staining of frozen and fixed sections of rat embryo spinal cords (Bahmanyar *et al.*, NEUROLOGY 1981). Already to our surprise the antibody has not been found in a large series of sera from patients with autoimmune

collagenous diseases which were positive for anti-rheumatoid factor and anti-DNA antibody. The possibility that these unconventional viruses use a filamentous cytoskeletal protein of the host in their structure as do some bacteriophages and plant viruses must be considered.

#### NEWLY EXTENDED RANGE OF CLINICAL DISEASE ASSOCIATED WITH CREUTZFELDT-JAKOB DISEASE DIAGNOSIS

In a paper in press in BRAIN (Masters, Gajdusek and Gibbs) we are presenting data of the transmission of spongiform encephalopathy to non-human primates inoculated with three atypical cases of CJD. They were atypical because of the presence of an unusually long course, the early clinical appearance of ataxia and other cerebellar symptoms, the very slow and only moderate degree of dementia, and neuropathologically revealing extensive distribution of amyloid plaques resembling those observed in kuru patients. These cases show a remarkably similarity both clinically and pathologically to New Guinean kuru much more so than does the more classical CJD patients we have studied. In an extensive review of the world literature we have found a large literature reporting this type of disease not usually diagnosed as CJD and often occurring in hereditary clusters. In such families many of the affected members have little or minimal dementia. Thus, the strong possibility that we must now search for the CJD virus in a wider group of patients than those with the presenile dementia of classical CJD has been demonstrated. Specifically, patients with spinocerebellar degeneration are called to question. In our report in press we are calling the cases comprising this syndrome, not previously brought together, the Gerstmann-Straussler syndrome.

#### LONG-TERM INCUBATION PERIODS OF KURU, CREUTZFELDT-JAKOB DISEASE AND SCRAPIE IN NON-HUMAN PRIMATES

The year-to-year surveillance of the occurrence of kuru in Papua New Guinea by direct clinical observation has shown that the incubation period in the human population at risk can be as long as 20 to 30 years following exposure. A recent analysis of our laboratory transmission data from non-human primates maintained longer than thought reasonable by investigators in the field of infectious diseases clearly supports the clinical observation made in New Guinea. Nine non-human primates developed experimental kuru following incubation periods which have ranged from 6 to more than 12 years. Of particular importance among this group were two chimpanzees that had been injected by peripheral routes only (iv, ip, sc, im) and a spider monkey which had been injected intracerebrally and intravenously with a pool of visceral tissues (liver, kidney, spleen) and developed disease 142 months, 82 months, and 123 months, respectively, following inoculation. Similar long incubation periods have been observed in animals inoculated with CJD infected tissues (72 months- 117 months) and scrapie infected tissues (72-74 months). In addition to the intracerebral route we have now conclusively demonstrated that these diseases can be transmitted by the following individual routes of inoculation: intravenous, intraperitoneal, subcutaneous, intramuscular, interdermal, intranasal, and oral. The later findings coupled with the extremely long incubation periods, particularly noted following peripheral inoculations since this is the most likely route of natural infections, have



great impact on our epidemiological studies and research into the etiology of other degenerative neurological diseases.

#### SUMMARY

The elucidation of the etiology and epidemiology of a rare, exotic disease restricted to a small population isolate--kuru in New Guinea--has now brought us to worldwide considerations that have importance for all of medicine and microbiology. For neurology, specifically, we have considerable new insights into the whole range of presenile dementias, and, in particular, to the larger problems of Alzheimer's disease, familial and senile dementias, and the processes of CNS aging. The implications of vertical transmission of slow virus infections, of conjugal transmission of these diseases, and of host genetic control of disease expression for all genetic diseases, and the relationship of these slow virus infection processes to those which may lead to neoplastic transformation are obvious.

The major problem among the degenerative diseases of multiple sclerosis, Alzheimer's disease, amyotrophic lateral sclerosis, and Parkinsonism remain unsolved, although there are tantalizing laboratory and epidemiological data pointing to the possible role of virus-like agents in these diseases. Perhaps the masked and defective slow infections with conventional viruses such as are seen in PML and SSPE may provide the best leads for studying these diseases.

#### AMYOTROPHIC LATERAL SCLEROSIS AND PARKINSONISM DEMENTIA IN HIGH INCIDENCE FOCI

Our scientific direction of the amyotrophic lateral sclerosis (ALS) studies at the Guam laboratory of NINDS for the study of the ALS-PD complex in high incidence among the Chamorro people, has resulted in some 12 publications which have already appeared, or are in press, and many promising ongoing studies. These are summarized below, but they indicate our conviction that the answer to the perplexing problem of motor neuron disease (ALS) and Parkinsonism-dementia (PD) are to be found in these ethnically and geographically limited foci.

Our study of the similarly intense focus of ALS and Parkinsonism and dementia among the isolated Jakai and Auyu people of Western New Guinea, discovered during our field studies (New England Journal of Medicine, 1963), and with two recently updated reports just published (Ciba Symposium, 1977; Symposium on ALS, February 2-3, Tokyo, 1978) (Neurology, in press) is proceeding with further field work this year. This year's work has proven that the disease is fully environmental and that ALS and PD are related as evidenced by (1) husband and wife with classical ALS; (2) husband with pure PD, wife with classical ALS, simultaneously; (3) next door neighbor to (2) above with classical PD; and (4) two women with classical ALS in 1974 in same village and a neighbor with PD. It appears that the "rule" is that people living or drinking exclusively from small springs and rivers originating in the "red-soil" lowland plain get ALS/PD. People of the same cultural and linguistic groups as these suffering from ALS and PD but living on tidal flats and on big rivers originating from the high mountains do not get ALS/PD. The water and soil analyses indicate extremely low calcium in garden soils and drinking water and the pattern of occurrences seem, as in endemic goiters to follow geological features of the environment rather than the patterns of ethnic and cultural demographic distribution. With this in mind, we are covering

possibilities of mineral metabolism, imbalances and trace metal toxicity as well as those of an endogenous virus in an isolated population in our studies on Guam and West New Guinea.

We have increased our collaborative research with the Japanese investigators, who have been helping us on Guam by providing us each year with a young neurologist to assist in the clinical neurological surveillance and care of our patients there and in collaborative pathological, biochemical and pharmacological studies. During this reporting period, Dr. Takao Makifuchi, of the Brain Research Institute, Niigata City, Japan, took up residence on Guam as a Visiting Scientist; and now Dr. Kiyomitsu Oyanagi has arrived to replace him. Also, Dr. Richard Yanagihara was recruited for Guam, and after three months of intensive preparation and developing protocols here at NIH proceeded to Guam where he initiated a study of Ca, P, Mg<sub>2</sub> and trace metal metabolisms including C47 calcium trace studies on ALS, PD and control subjects.

The Japanese are themselves concerned with their own foci of high incidence of ALS and PD on the Kii Peninsula of the main island of Japan. The series of meetings and conferences on ALS in Japan held in March 1978 resulted in the confirmation by Dr. Hirano of the pathological identity of the Kii Peninsula PD cases with those on Guam (both demonstrating neurofibrillary tangles), and the final agreement that the two disease foci represent the same disease complex. During his 1979 field studies in West New Guinea, the Chief, LCNSS, has obtained definitive evidence that classical Guamanian ALS, PD, and ALS/PD does occur in the high incidence foci he discovered in West Irian and is very excited about resolving this problem. In addition, Dr. Gajdusek noted the occurrence in West New Guinea of a subacute progressive paralysis that looks like "slow-poliomyelitis" vitamin B deficiency. He has seen many cases this year and recognized it as the same disease he first saw in 1974-1976 field trips. The disease is not ALS; it can be "acute", it is often fatal, but remissions and recurrences do occur. A few cases have had beriberi-like edema with onset but most have not. That this very severe paralytic disease should occur within the ALS/PD focus is amazing. International collaboration and, most importantly, more original and innovative research concepts and more imaginative and cautious study of the various Western Pacific foci have continued and been expanded. Those studies which are underway in our collaborative project, and a bibliography of recent publications (1975-1980 in press) resulting from studies of these foci are included as an appendix to this annual report. The ongoing studies include:

- (1) Clinical variations in ALS-PD complex in Chamorros;
- (2) Human biology of ALS-PD complex and other chronic diseases in Chamorros of the Mariana Islands;
- (3) Chronic CNS disease and disability survey of Guamanian Chamorro migrants to the mainland United States;
- (4) Genetic studies of the Chamorro population, both normal and ALS-PD afflicted;
- (5) Detection of sedimentable reverse transcriptase activity in the brains of patients dying with ALS-PD;
- (6) Search for biochemical defects in ALS-PD brains by gel diffusion chromatography;
- (7) Search for nucleic acid repair mechanism defects in transformed leucocyte cell lines derived from ALS-PD patients;



- (8) Search for an ALS or PD specific antigen in brain tissues by clonal myeloma cell hybridization with spleen cells of ALS and PD from hyperimmunized animals and resultant monoclonal antibody production;
- (9) Trace aluminum and other heavy metal studies in brain, CSF, blood and other tissues of ALS-PD patients;
- (10) Evaluation of the precise nature of the cognitive and affective defects and the progression of dementia in the PD patient;
- (11) Evaluation of liver function and pathology;
- (12) Development of techniques for the unmasking of an infectious agent by in vitro techniques;
- (13) Assessment of the immunological competence of patients;
- (14) Attempts to transmit ALS-PD to non-human primates and non-primate hosts;
- (15) Major virus group seroepidemiology of the Mariana and Caroline Islands, Japan, and West New Guinea populations with relation to ALS-PD;
- (16) Pharmacologic studies of ALS-PD;
- (17) Elucidation of osteoporosis, osteoarthritis, and bone deformities in the Chamorros; and
- (18) Evaluation of the growth and development of normal Guamanian children and adolescents--a 30-year follow-up study.

The genetic studies, already well advanced, include blood group factors, red cell enzymes, serum proteins, HLA typing, and mixed leucocyte agglutinins, dermatoglyphics, anthropometry and other gene markers.

#### Epidemiology of ALS and PD in Migrants to and Immigrants from Guam

Since World War II, there has been an extensive migration from Guam of at least 15,000 Chamorros, primarily to the United States. This represents nearly one-third of the total Chamorro population of 47,000 residing on Guam. Amyotrophic lateral sclerosis has developed in 14 Chamorro migrants from Guam to the United States, Japan and Korea after periods of one to 36 years of absence from Guam. Nine of these cases have been previously reported. In another eight subjects ALS has developed within 1 to 14 years of their return to Guam after absences of many years from the islands. Parkinsonism dementia, a high incidence presenile dementia peculiar to Chamorro Guamanians, has developed in one subject 46 years after his departure from Guam. It appears that the onset of ALS in these patients after long absences from Guam will demonstrate the lower limit for the incubation period in each case if a toxic or infectious exposure occurring only on Guam is the cause of the disease.

Additionally, during the past two decades there has been an increasing number of cases of Guamanian ALS in long-term Filipino migrants to Guam. The average annual incidence rate of ALS in these migrants is approximately five-fold higher than the rate of ALS in the United States. Parkinsonism dementia-like disease has been clinically identified in five Filipino patients and one case with autopsy verified pathologically. Because of the high degree of genetic similarity between the Chamorro and Filipino peoples, which we have recently demonstrated, a detailed epidemiological survey for ALS and a clinical search for PD in the Phillipine Islands is currently being conducted by members of this laboratory.

The clinical and pathological characteristics of long surviving cases of Guamanian ALS, that is of more than ten years duration, are currently under

study. Long surviving cases of ALS in Guam are younger, have a familial occurrence, have a different sex ratio, and show a different pattern of disease progression than those with a normal duration of disease.

### Immunology of ALS and PD on Guam

Additional studies on HLA, dermatoglyphics and other gene markers, on osteoporosis and osteoarthritis, on heavy metals and other environmental toxins and on a ten-year follow-up study of the descriptive epidemiology of ALS and PD are close to completion. Further studies based on these data are in the planning stages or already underway.

Previous studies in our laboratory have shown that ALS and PD patients from Guam had diminished levels of cellular immunity as determined by diminished response to skin test antigens, lymphopenia, diminished number of 'T' cells, and decreased mitogenic response, than those of age- and sex-matched Guamanian controls. Further, ALS patients with HLA BW-35 had diminished cellular immunity and shorter mean duration of the disease. This association was found to a lesser degree among PD patients and no association was detected in the controls. Using C19 binding techniques, Oldstone *et al.* have shown high frequency of immune complexes in the sera of ALS patients in the continental United States. There was evidence of immune complex deposition in some of the kidneys of the ALS patients. The nature of these immune complexes was not determined. Studies of hepatitis B in the South Pacific reveal that hepatitis B virus is endemic in most of the Pacific Islands. There is high prevalence of hepatitis B surface (HBsAg) antigenemia, and most of the population has either HBsAg or antibody to HBsAg. It is common to have found both HBsAg and anti-HBsAg in many individuals in the population. Since immune complexes are known to cause immunosuppression, we investigated the prevalence of HBsAg, anti-HBsAg, and the immune complexes due to HBsAg and anti-HBsAg in the sera of ALS and PD patients from Guam and healthy controls. Additionally, we also tested sera for the presence of hepatitis A antibody. The data showed that ALS patients have lower levels of anti-HBsAg than PD patients or controls. There was no significant HBs antigenemia or immune complexes in ALS and PD patients and controls. Almost all sera tested had antibodies to hepatitis A. These studies show that HBsAg and anti-HBsAg complexes were not responsible for the immunosuppression observed. The lower rates of HBsAg in this population may be due to sampling of older individuals.

In other areas of Micronesia, human biological field and laboratory studies continue. Studies of chronic respiratory diseases indicate that 75% of the children under five years of age were found to have asthma, while over 50% of the adults over 40 years of age were affected by chronic bronchitis, often with an asthmatic component, and typical chronic obstructive airway disease occurred in almost one-third of the male population over 50 years of age. As a result, pulmonary airway diseases constitute the most important cause of morbidity and mortality in the Western Caroline Islands.

### CHRONIC ENCEPHALITIS AND EPILEPSY

Since chronic inflammatory neurological disease is known to follow togavirus (arbovirus) encephalitis infections of humans in Europe and Asia, sera from more than twenty American patients with chronic epilepsy and inflammatory brain disease were examined by hemagglutination for all togaviruses known to cause

encephalitis of humans in North America. None had antibodies. It seems unlikely that togavirus encephalitis is an important cause of chronic inflammatory brain disease in the United States.

A survey of togaviral antibodies in several Pacific populations confirmed earlier studies of the geographic distribution of several viruses. A possible correlation between susceptibility to Ross River Virus and one red cell Rh subtype was found in a population of Papua New Guinea. Plaque and microtiter tests have been developed for groups A and B togaviruses, and neutralization tests are being performed on selected sera.

#### SCHIZOPHRENIA AND JUVENILE AUTISM

Serum and CSF specimens from schizophrenic patients and age- and sex-matched controls were obtained from Doctors Torrey and Wineberger of St. Elizabeth's Hospital, Washington, D.C. and Constantine Sakkles of the University of Maryland Hospital, Baltimore. These specimens were tested for group A and group B arboviruses using the hemagglutination inhibition test. Viral antigens used in the test were Eastern and Western Encephalitis, St. Louis encephalitis, and California encephalitis. There was no significant association of arboviral antibodies to schizophrenia. In the light of recent reports by Tyrell *et al.* of detection of cytopathic agents from the CSF and some controls, attempts will be made to do similar studies with the CSF samples on hand.

The work on the development of animal models for the study of persistent infections has continued. A foamy virus of chimpanzees (Pan 1, also called foamy virus 6) was isolated in this laboratory over ten years ago. In the chimpanzee it appears to be a latent virus, and can at times be isolated from brain explants of healthy animals. The mechanism of viral latency has been impractical to examine, however, due to the expense and scarcity of the chimpanzee for experimental purposes. Therefore, experiments were conducted to adapt Pan 1 virus to a more convenient laboratory host, and after several preliminary studies, we succeeded in adapting the virus to the mouse. Using kidney and spleen explants from mice-infected neonatally, infectious virus has been isolated up to one month following inoculation, viral antigen has been demonstrated in the explants, and serum CF antibody has been detected. However, in no animal has it been possible to detect infectious virus or viral antigen directly in the organs themselves. We are currently studying the possibility of viral persistence for up to a year following inoculation, and evaluating the mice for any signs of disease during their natural lifetime. Integration of viral genome in the host cells is also under investigation in collaboration with Dr. Chev Kidson in Australia.

The model of lysogenicity and of subviral genetically active macromolecular structures from the study of bacterial viruses and bacterial genetics supply ample imaginative framework for an expression of our ideas of possible pathogenic mechanisms for kuru and CJD in man. The unconventional viruses of the spongiform encephalopathies tax even our imagination in relation to molecular biology gained from these studies in bacteria.

For a now-disappearing disease, kuru, in a small primitive population to have brought us this far is ample reason for pursuing intensively the challenges offered by the still inexplicable high incidence and peculiar profusion of different neurological syndromes, pathologically distinct yet apparently related



to each other, which have been discovered in the several small population enclaves we have investigated. Thus, the high incidence of ALS, ALS-PD on Guam and among a small population of people in West New Guinea, coupled with the high incidence of ALS on the Kii Peninsula of Japan, may indeed profusion of different neurological syndromes, pathologically distinct yet apparently related to each other, which have been discovered in the several small population enclaves we have investigated. Thus, the high incidence of ALS, ALS-PD on Guam and among a small population of people in West New Guinea, coupled with the high incidence of ALS on the Kii Peninsula of Japan, may indeed offer the best opportunity of solving the problem of this sclerosing disease which in the United States has an incidence as high as that of multiple sclerosis.

The delineation of infection as the etiology of heredofamilial and presenile and senile dementias of man was made possible only through the concomitant studies on the neurobiology of population isolates. In this area we have been engrossed in the investigation of deaf-mutism, mental subnormality and other congenital central nervous system defects associated with endemic goiter in the Central Highlands of Western New Guinea, as well as patterns of delayed puberty, slow growth rates, and of early aging in isolated Melanesian groups. Ethnic drug abuse (particularly of kava), strange patterns of psychosexual development, pseudohermaphroditism, and culturally-determined responses to pain, and roots of aesthetic expression, have all been under study. Foci in primitive population isolates of familial periodic paralysis, progressive muscular dystrophy (both the pseudohypertrophic type of Duchenne and the non-pseudohypertrophic distal type), amyotrophic lateral sclerosis and Parkinsonism, are also being investigated. Genetic studies on human evolution led to the discovery of new genetic factors among haptoglobin, hemoglobin, and red cell enzyme pleomorphisms and the definition of their biochemical structure.

#### A NEW FORM OF CYSTICERCOSIS EPILEPSY IN MAN AND A NEW SEROLOGICAL TEST FOR CYSTICERCOSIS

The further significance of scientific investigations of small population enclaves of remote populations was even more dramatically apparent during recent field trips of the Chief of LCNSS, with his re-evaluation of what may turn out to be one of the largest "epidemics of epilepsy" ever recorded. This continues to occur in the Wissel Lakes area of West New Guinea and is the result of cysticercosis, an infestation with the larvae of Taenia solium, the pig tapeworm, newly introduced into New Guinea. Our recent studies have led us to conclude that the natural history of this cysticercosis epilepsy is not a result of death of the worm, scarring and calcification of lesions, as much of the literature suggests, but is an early sign of inflammation from new invasion of the brain by the Taenia larvae. After one, two or three grand mal seizures no further convulsions occur and most patients are left without sequellae. Two patients who have died had the most heavily infected brains ever seen, still had fresh uncalcified cysts, further confirming the thesis that the self-limited seizures result from primary invasion of the larvae and not from old calcified cysts breaking down. Convulsions often occur even before the first subcutaneous nodules appear, and as the nodules increase in number, additional seizures occur. The high incidence of severe third-degree burns, which may even result in death, is a direct result of cysticercosis-induced seizures that occur during sleep, throwing the patient into the house fire. The unclothed people, living at a 2000 meter elevation, need to sleep close to the home fires on cold nights. We are able to date the first introduction of Taenia solium into the area and to

plot the spread of taeniasis in pigs and man, and of cysticercosis and associated epilepsy in man, to other previously Taenia-free areas. During this year, we have learned that the cysticercosis has spread both in swine and man throughout the West New Guinea Highlands and is now in the Baliem region. With Dr. Budi Subianto, the local Indonesian medical officer, a visiting scientist in our laboratory, we have planned a neuroepidemiologic study aimed at elucidating the natural history of the epilepsy and acute psychoses and other neurological complications that have occurred concomitantly with the emergence of subcutaneous cysticercosis nodules.

Recently, we developed an enzyme-linked immunoabsorbent (ELISA) serological test for diagnosis and seroepidemiological surveillance of cerebral cysticercosis. Sera collected from adjacent populations prior to the introduction of *T. solium* and in 1974 and 1977 from patients with epileptic seizures, subcutaneous nodules, and other manifestations of cysticercosis at the Enarotoli hospital were studied. Positive control sera and cerebrospinal fluid (CSF) were from patients with neurocysticercosis in Mexico: their clinical disease had been previously confirmed by the presence of complement-fixing antibodies to cysticercus antigens. For the ELISA test cysticercus antigens were high speed supernatant of a sonicated 20% suspension of cysticerci dissected from Balinese pigs killed in Jakarta; control antigens were similarly prepared from normal pig tissues. The ELISA procedure was that of Voller and Bidwell (1975) and Yolken et al. (1977) for rota virus assays. Titers were expressed as ratio of highest dilution of serum bound by cysticercus antigen to that bound by control antigen of same protein content. Standardization was done using antisera prepared in rabbits injected with cysticercus antigen in complete Freund's adjuvant. In symptomatic patients 5 of 6 (83%) with skin nodules, 7 of 9 (78%) with convulsions and skin nodules, and 7 of 16 (44%) new epileptics without skin nodules had antibody while among non-symptomatic residents of the Wissel Lakes area 4 of 52 (8%) had antibody. None of the 281 sera collected from people outside of the Wissel Lakes area had cysticercus antibody. Among the specimens from Mexican patients with neurocysticercosis 11 of 14 (79%) of the sera and 20 of 25 (80%) of CSF had antibody with geometric mean titers of 580 and 1600, respectively.

Higher percentage of positive patients with systemic cysticercosis may possibly be due to exposure to a larger antigenic mass. The lower positive rates observed among cerebral cysticercosis patients may be due to lack of antibody response due to direct massive infection of the brain by the parasite and short incubation period prior to detection of convulsions. The importance of cerebral cysticercosis in the third world countries cannot be underestimated. The ELISA test provides a simplified sensitive technique adaptable to field use for determining the presence and magnitude of human infections with cysticercus. However, because of its sensitivity cross reactivity has been observed to occur with antibodies to other parasitic diseases. This has led the studies on the development of techniques to produce purified cysticercosis antigens for enhancement of the specificity of the reactions.

#### VILYUISK ENCEPHALOMYELITIS IN IAKUT PEOPLE OF THE SOVIET SIBERIA An Old Chronic Infective Degenerative Disease of the CNS New to Western Medicine

As previously reported, the Chief of LCNSS was invited by the Soviet investigators to participate in the investigations in the U.S.S.R. of a unique degenerative disorder of the nervous system, Vilyuisk encephalitis. This



disease occurs only in the Yakut region of Eastern Siberia and has many features of a slow virus disease. In 1978 he finally saw and examined patients flown to Moscow. In August 1979 a field study in Yakutia was completed, the first by any western investigator, and many patients with VE were seen throughout the Yakut area. Pathological specimens have been obtained and an extensive case records and photographic documents are being analyzed. The diseases of Siberia and the last two decades of Soviet work on the disease, which is clearly infectious, were reviewed. We shall continue our collaborative study of this disease with our Soviet colleagues and we are in the process of writing for publication extensive reports on our field studies and laboratory investigations.

SPINOCEREBELLAR DEGENERATIONS IN HIGH INCIDENCE IN YAKUT PEOPLE OF SOVIET SIBERIA AND IN LES PETIT BLANCS DES HAUTS OF ILE DE LA REUNION, INDIAN OCEAN  
HEMORRHAGIC FEVER WITH RENAL SYNDROME

In 1981 the Chief of LCNSS completed a second field visit to Ile de la Reunion in the Indian Ocean where we have encountered foci of high incidence spinocerebellar diseases, including a variant of Friedreich's ataxia, another of Marie's spinocerebellar degeneration, and a third of Ramsey Hunt disease occurring exclusively in the "les petits blancs des hauts", very highly inbred descendants of the first French settlers on this previously uninhibited island some three centuries ago.

Among Yakut people of Soviet Siberia there is a huge collection of genetically determined Marie's type of spinocerebellar degeneration which we (DCG) have had a chance to see and study in the field with Dr. Prokopii Petrov and Dr. Lev Gertsovich Goldfarb.

In view of the transmissibility to laboratory primates of familial, apparently dominant genetically determined forms of CJD and of the Gerstmann-Strausler syndrome, we are very interested in these other spinocerebellar degenerations. They are being studied for possible transmissibility and from the possibility of providing a series of pleomorphic alleles determining cerebellar degenerations of differing forms at various times of life. We hope to parallel some of the studies of the Barbeau Canadian group studying Friedreich's ataxia in Quebec, which differs somewhat clinically from the syndrome on la Reunion.

During the period covered by this report significant progress has been made on our studies begun in 1953 on the hemorrhagic fevers with renal syndrome that severely affected United Nations troops during the Korean War and for which an etiologic agent had not been isolated in spite of enormous efforts on the part of the Walter Reed Army Institute of Research of which we were then a part. The isolation by Lee and Lee in 1978 of the viruses responsible for HFRS has provided us the opportunity to reinvestigate this disease, characterize the virus and carry out collaborative studies with colleagues in China, the USSR, Finland, Sweden, Yugoslavia, Japan and Korea. In our first review of hemorrhagic fever with renal syndrome Gajdusek in 1953 indicated that clinical severity, particularly hemorrhagic manifestations, of this chronic viral nephropathy varies from one geographic region to another. We suggested that nephropathia epidemica (NE) of Scandinavia was a mild form of HFRS or Korean hemorrhagic fever (KHF) with no or very minimal hemorrhagic manifestations. Mortality in the Far East (China, Korea, USSR) ranges from 5-30%, in European USSR it is lower, while NE is rarely fatal. The sylvatic reservoir for the

virus in Scandinavia and European USSR is in wild voles (Clethrionomys sp.), whereas in Eastern Asia it is in the field mouse (Apodemus agrarius). The rat, Rattus rattus, appears to be the reservoir in Japan and in urban foci in Korea. Laboratory rats in Japan and Belgium are infected and have caused HFERS in laboratory workers. The seasonal occurrence varies. Thus, cases are most frequent in the late fall and winter in Scandinavia at a time when wild voles enter dwellings and graineries. In southern and central China cases are more frequent in the autumn, during threshing season, and epidemiology has incriminated the respiratory route of infection. In both East and West sporadic cases occur yet epidemic outbreaks are frequent. This seems to be determined by the particular circumstances of exposure to the rodent reservoir. The military experiences in the Soviet Far East, Manchuria, and Korea of the Russian, Japanese, and United Nations armies, respectively, indicated two epidemic peaks, the first in late spring and early autumn, and the second in late summer and early fall; this was taken to suggest mite- or chigger-borne infection, as is the case with Tsutsugamushi disease. Lee, however, has not found virus in ectoparasites collected from infected rodents. The virulence, as evidenced by hemorrhagic manifestations, systemic reaction and mortality varies as one moves from Far Eastern Asia to eastern and northern Europe. This parallels the shift of virulence of tick-borne encephalitis across the Eurasian land mass. However, Japanese cases are less severe, resembling NE more than KHF; possibly, the virus in rats is less virulent for man. Detailed serological comparisons of strains isolated in different regions are necessary to establish the closeness or divergence of the etiological viruses in various foci, and recent adaptations of the virus to laboratory rats, athymic nude mice, and tissue culture have now made this possible.

The first clear-cut evidence that hemorrhagic fever with renal syndrome virus infections were occurring by the respiratory route stems from the large outbreak of laboratory infections in Moscow in 1962 with 83 affected laboratory workers. A more recent epidemiological study of infections in medical research laboratories in Japan and Belgium have indicated a respiratory route of infection of laboratory workers working in animal experimental rooms in contact with enzootically silently infected commercially reared white rats. Epidemiological studies in outbreaks in China (Xu et al., 1979) also led to the conclusion that most infection was by contaminated aerosols. Clinical and epidemiological studies in Scandinavia, Hungary, the Soviet Far East and Korea failed to directly incriminate the respiratory route of infection. But exposure to urine and feces contaminated foodstuffs and aerosols, or anthropod vectors, and ectoparasites such as mites and chiggers, on infected rodents were usually thought to be the source of human infection. However, it is now evident that infection occurs most often by the respiratory route from contaminated aerosols produced by the asymptotically infected reservoir rodents. Whether saliva and respiratory droplet infection--the only secretions from which virus has been isolated--is the only source of such aerosol contamination remains to be proved. Finally, high titer antigen has been found only in the lungs in infected wild mice (Apodemus agrarius), voles (Clethrionomys glareolus), wild urban rats (Rattus rattus), and laboratory rats of the Wistar strain in Japan; other tissues contain less concentrations of antigen as demonstrated by immunofluorescence. In experimentally infected white rats (Wistar and Fischer strains) and athymic nude mice the virus also appears in highest concentration in the lungs. The virus has to date been isolated only from lung, saliva, throat washings and blood of human patients, and no other tissue or secretion has yet been found to be infectious. In naturally and experimentally infected

rodents the virus has not to date been isolated from feces or urine, but it has been obtained regularly from lung, saliva, and acute phase blood.

Until recently, the serological relationship between Scandinavian nephropathia-epidemica (NE) and Korean hemorrhagic fever (KHF) has been established (Svedmyr, 1978; Lahdevirta, 1979). This was first done using only as antigen KHF virus propagated in the lungs of naturally and experimentally infected *Apodemus agrarius* mice. We have recently confirmed this antigenic relationship by demonstrating specific neutralizing antibody to KHF virus in convalescent sera from patients with NE. Similar relationships have been shown for HFRS in European Russia with KHF virus. However, until the European virus was isolated from NE in Finland, it was previously impossible to check for immunological crossings in both directions. This has now been done and it is clear that NE sera react with Korean antigen in the immunofluorescent tests at almost the same titers with the homologous antigen from naturally infected or experimentally infected *Clethrionomys* lung. KHF human sera, on the other hand, give much higher titers with the homologous Korean virus in *Apodemus* lung than with the Finnish virus in *Clethrionomys* lung. Sera from patients convalescent from HFRS in southern and central China react by immunofluorescence similarly to KHF sera as sera from HFRS patients in the Soviet Far East and in Japan. All these Asian sera (Chinese, Soviet, Korean and Japanese) from HFRS patients as well as Scandinavian NE sera neutralize several logs<sub>10</sub> of KHF virus but qualitative cross neutralization tests have not yet been possible since the NE agent is only propagated with difficulty in *Clethrionomys* voles. Thus, the serological crossing is a partially one-way cross, with KHF sera reacting at 10- to 20-fold lower titer with NE antigen than with the homologous antigen, while, in contrast, Scandinavian NE sera show only a 2-fold reduction in titer with lung from *Apodemus* or nude mice infected with KHF than with the homologous antigen in *Clethrionomys* lung. Where in crossing Soviet Eurasia the shift to the NE from the KHF serological type occurs, remains to be determined. Sera from Balkan (Czechoslovakia, Hungary, Bulgaria, Rumania, and Yugoslavia) cases of HFRS are now available for such study. We have demonstrated closer serological relationships with NE than with KHF in Yugoslavia sera from HFRS patients, in keeping with the geographic shift of the serotype from Asia to Europe.

In a previous report (XIV Pacific Science Congress, 1979) we conjectured about the possible presence of unrecognized hemorrhagic fever with renal syndrome (HFRS) in North and South America and other areas of the world wherein the disease had not previously been recognized. The natural host of HFRS in northern and eastern Europe, *Clethrionomys* sp., is indigenous across northern North America in Canada and the United States from Maine to Alaska. The murine host of the virus of Korean hemorrhagic fever (KHF), *Apodemus* sp., is not found in the Americas. *Clethrionomys*-borne disease in Europe has proved to be less severe clinically, and demonstrates fewer hemorrhagic symptoms than the *Apodemus*-borne disease in eastern Asia (China, USSR, and Korea). Thus, a milder form of nephropathy associated with little or no hemorrhagic diathesis, as in nephropathia epidemica (NE) in Scandinavia, might be expected in the Americas. Using the indirect immunofluorescence test for demonstrating specific antigen-antibody reactions in KHF infections we have tested sera from Alaska, South America, Iran, and India. In the first 100 sera we studied from Alaska we reported no antibodies to KHF virus; however, when this series was extended to 600 specimens a single serum had specific antibody to KHF virus at titer 1:128. We also tested 4 cerebrospinal fluids (CSF) and 16 convalescent sera from



children with an undiagnosed acute febrile illness in Santa Cruz, Bolivia. Although none of the 4 CSF reacted, 2 of the 16 sera had antibody titers to KHF virus of 1:256 and 1:128, respectively. Of 251 sera from residents of remote rural villages in India, 2 had antibodies to KHF virus; a 35-year old male gardener and a 27-year old female with titers of 1:256 and 1:640, respectively. Casals has found (personal communication) that high titering specific antibody to KHF virus failed to react in the HAI test against Japanese B, Murray Valley, Omsk hemorrhagic fever, and Chikungunya antigens. We found that high titering rabbit antisera or mouse ascitic fluids to more than 30 arboviruses, including Rift Valley fever and Junin viruses, and antisera to simian hemorrhagic fever virus did not react with KHF virus in the IF test. No other viruses are known to cross react with HFRS by the IF test. Neutralization tests on the few positive sera we have found from Alaska, Bolivia and India are in progress. These preliminary data suggest a possible wider distribution of HFRS viruses than is now known and further seroepidemiological screening from other parts of the world is clearly needed.

#### GENETIC EFFECTS ON SUSCEPTIBILITY TO ARBOVIRUS INFECTION

Continuing our more than three decades on work on the arthropod-borne viruses we have this year completed a study on human variation and infection with these viruses in humans in New Guinea. Antibodies to group A (Chikungunya, Getah, Sindbis, Ross River) and group B (dengue 2 and 4, Murray Valley encephalitis, Japanese encephalitis, Yellow fever, Zika) arboviruses were measured by hemagglutination inhibition and neutralization in sera from selected aboriginal populations of New Guinea. Antibodies to Murray Valley encephalitis and Ross River viruses were highly prevalent in most of the lowland populations. For each population the presence of antibodies was correlated with 12 genetic polymorphic systems: 7 blood groups (ABO, MN, Ss, Rh, P, Kidd, Duffy), 3 red cell enzymes (acid phosphatase, 6-PGD, PGM), and 2 serum proteins (haptoglobin and immunoglobulin Gm).

There were no significant associations between any marker system and Murray Valley encephalitis virus infection. For one population, two blood group systems, Rh and Kidd, showed statistically significant associations with antibodies to the Ross River virus. Among individuals with the Rh phenotype  $R_1R_0$  (CcDee), the relative risk of infection with Ross River virus was five times less than that for other members of the population. The relative risk of Ross River virus infection in individuals with Kidd phenotype Jka- was approximately three times less than that of the Jka+ individuals.

The reasons for those associations are unknown. Hypothetical explanations include differences in cell membranes of some Rh and Kidd phenotypes impeding attachment of virus, hereditary impairment of immune responses to the virus, shared antigens between the virus and blood-group substances resulting in immune tolerance, and decreased biting by mosquitoes of individuals with particular phenotypes. It is also possible that some genetically related social subgroup of people with less exposure to mosquitoes exists in the population. The associations between Rh and Kidd phenotypes and susceptibility to group A or other arthropod-borne infections must be confirmed by studies of larger populations living where such infections are endemic.

The development and maturation of the two major projects of this laboratory has resulted from cross-fertilization of each since their origin, and both have

grown from the basic studies on child growth and development and disease patterns in primitive cultures. Although the two projects, each composed of many subsections, differ markedly in the questions they address and the techniques of investigation they employ, much of the field data collected from one project is also requisite for the studies in other projects. Both are served by the same investigators, who function as a team. These scientists derive their creative stimulus, dedication and enthusiasm to a great extent from the atypical and exotic biological, social and cultural materials presented, and the diverse, frequently unconventional, approaches of the two projects.

Principal Investigators: D. Carleton Gajdusek, M.D.  
Clarence J. Gibbs, Jr., Ph.D.  
Paul W. Brown, M.D.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)		U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 01282-17 CNSS
PERIOD COVERED October 1, 1980 through September 30, 1981			
TITLE OF PROJECT (80 characters or less) Neurobiology of Population Isolates: Study of Child Growth and Development, Behavior and Learning, and Disease Patterns in Primitive Cultures			
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PRINCIPAL INVESTIGATORS: D. Carleton Gajdusek, M.D., Chief, LCNSS; and Clarence J. Gibbs, Jr., Ph.D., Deputy Chief, LCNSS; David M. Asher, M.D., Paul W. Brown, M.D., and Ralph M. Garruto, Ph.D.  OTHERS: Michael Alpers, M.D.; Judith Farquhar; Peter Fetchko, M.A.; Dmitry Goldgaber; Klaus Mannweiler, M.D.; Steven Ono; Robert G. Rohwer, Ph.D.; Donald Rubinstein, Ph.D.; Vincent Zigas, M.D.; Francoise Cathala, M.D.; Kwang-Ming Chen, M.D.; Olivia Cruz, M.D.; Richard Feinberg, Ph.D.; Robert MacLennan; Father David Gallus; Fusahiro Ikuta, M.D.; Jesus Raglmar; John Runman; Marie-Claude Moreau-Dubois, Ph.D.; Millicent Coker-Vann, Ph.D.; Mario Barragan, M.D.; Sina Bahmanyar, M.D.; Andres Salazar, M.D., Chen-ting Chin, M.D.			
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LAB/BRANCH Laboratory of Central Nervous System Studies, Intramural Research Program			
SECTION			
INSTITUTE AND LOCATION National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205			
TOTAL MANYEARS: 12	PROFESSIONAL: 8	OTHER: 4	
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER <input checked="" type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS			
SUMMARY OF WORK (200 words or less - underline keywords) Studies of human biology of vanishing primitive societies focus on neurological development and learning patterns in diverse cultural experiments in the human condition found in such isolated groups. Laboratory techniques of molecular biology, immunology, virology, endo-crinology and biochemistry in these cultures and field epidemiological, genetic isolated primitive bands than in civilized societies. Data and specimens collected over years on expeditions to Micronesia, Polynesia, Solomon Islands, New Hebrides, New Guinea, Indonesia, S. America, Asia and Africa are used. Studies on nutrition, reproduction, fertility, neuroendocrine influences on age of sexual maturation and aging, genetic polymorphisms, genetic distance, unusual and odd employment of the higher cerebral CNS function of language learning, cognitive styles, computation (calculation without words or numbers) and culturally modified sexual behavior elucidate alternative forms of neurologic functioning for man which we would be unable to investigate once the natural cultural experiments in primitive human isolates were amalgamated into the cosmopolitan community of man. Foci of high incidence prevalence of kuru, ALS/PD, epilepsy, other neurological degenerations, hysterical disorders, schizophrenia, neoplasms, goiter, cretinism, rheumatoid diseases, diabetes, asthma, chronic lung disease, malaria, filariasis, leprosy, cysticercosis and other infections are investigated.			

## COOPERATING UNITS: continued

Dr. Louis Herzberg, Perth Medical Center, Nedlands; Dr. Chev Kidson, Queensland Institute of Medical Research, Brisbane; Dr. Robert L. Kirk, Australian National University, Canberra; Dr. Robert MacLennan, University of Sydney, Sydney; Dr. Colin Masters, University of Perth, Perth; Dr. John Sheridan, Queensland Institute of Medical Research, Herston; Dr. Fiona Stanley, Perth Medical Center, Nedlands; Dr. Neville Stanley, University of Western Australia, Nedlands; Dr. Stephen Wurm, Australian National University, Canberra.

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ITALY: Marek and Allison Jablonko, Perugia.

KENYA: Dr. Leendert C. Vogel, University of Nairobi, Nairobi.

MEXICO: Dr. Reinhart Ruge, Cernavaca.

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NETHERLANDS: Father Ben van Oers, Missiehuis, Tilburg; Dr. Jaap Goudsmit, University of Amsterdam.

NEW HEBRIDES: Capt. John Barley, Treasury/Customs Department, Port Vila; Dr. Kirk Huffman, Cultural Centre, Port Vila; Dr. Rabi Ramdoyal, World Health Organization, Port Vila; Dr. Ratard, French Hospital, Port Vila.

NEW ZEALAND: Dr. R.W. Hornabrook, Wadestown.

PAPUA NEW GUINEA: Dr. Michael Alpers, Institute of Medical Research, Goroka; Dr. H.A. Brown, Port Moresby; Rev. F. Fischer, Lutheran Mission, Okapa; Dr. J. Linsley Gressitt, Wau Ecology Institute, Wau; Richard Lloyd, Summer Institute of Linguistics, Aiyura; Mr. Ivan Mbagintao, J.K. McCarthy Museum, Goroka; Dr. Stuart Merriam, Highland Christian Mission, Yagusa; Dr. Jack Onno, Department of Public Health, Port Moresby; Dr. Kerry Pataki-Schweizer, University of Papua New Guinea, Port Moresby; Euan Scrimmour, University of Papua New Guinea; Dr. Alan Tarutia, Public Health Headquarters, Konedobu; Dr. Jeffrey Tuvi, Boroko.

PERU: Dr. Carlos Monge, Universidad Cayetano Heredia, Lima.

PHILIPPINES: Dr. Benjamin Catubay, Provincial Health Officer, Ilocos Norte; Dr. Martesio C. Perez, University of Philippines, Manila; Dr. Virginia Basaca Sevilla, Ministry of Health, Manila; Dr. Elizabeth Zaraspe-Yoo, University of Philippines, Manila.

MADAGASCAR: Dr. Pierri Coulanges, Institute Pasteur de Madagascar, Antananarivo.

MAURITIUS: Dr. B. Gurburram, Ministry of Health, Port Louis.

REPUBLIC OF CHINA: Dr. R. Palmer Beasley, University of Washington, Taipei.

REUNION ISLAND: Dr. Charles Bosquet, Hopital de Terre Rouge, St. Pierre; Dr. Jean-Baptiste Dandelot, Hopital de Terre Rouge, St. Pierre; Dr. Maurice Jay, Hopital Psychiatrique, St. Paul.

SCOTLAND: Dr. Alan G. Dickinson, A.R.C. Animal Breeding Research Organization, Edinburgh; Dr. J.D. MacGregor, Shetland Health Board, Shetland.

SINGAPORE: Chong Keat Lim, Architects Team 3; Prof. Dr. Lim Kok Ann, Dr. Ivan Polunin, University of Singapore; Dr. Foo Keong Tatt, Singapore General Hospital.

SOLOMON ISLANDS: Dr. D. Mackay, Center Hospital, Honiara; Dr. A.M.O. Solomon, Health Department, Kirakira; Dr. B. Wilkin, Central Hospital, Honiara.

SWITZERLAND: Dr. Liana Bolis, World Health Organization, Geneva; Dr. Stephen Fazekas, Basel Institute for Immunology, Basel.

## COOPERATING UNITS: continued

USSR: Dr. Mikhail Petrovich Chumakov, Institute for Poliomyelitis and Virus Encephalides, Moscow; Dr. Lydia L. Fadeeva, Ulitsa Valters Ulbrichta, Moscow; Dr. L.G. Goldfarb, Institute of Poliomyelitis and Viral Encephalitides, Moscow; Prof. Vera I. Il'yenko, All-Union Research Institute of Influenza, Leningrad; Miss Bela Kaplan, Institute of Poliomyelitis and Encephalitides, Moscow; Prof. D.K. Lvov, D.I. Ivanovskii Institute of Virology, Moscow; Dr. Prokopii Andrevich Petrov, Iakut Ministry of Public Health, Iakutsk; Dr. Anatoli Alexandrovich Smordintsev, Leningrad; Dr. Victor Zhadanov, Ivanovskii Institute of Virology, Moscow.

UNITED STATES: Alabama--Dr. James Dutt, University of South Alabama, Mobile; Dr. Charles Hoff, University of South Alabama, Mobile; Dr. Wladimir Wertelecki, University of South Alabama, Mobile; Arizona--Dr. Tim Kuberski, National Institute of Arthritis, Metabolism, and Digestive Diseases, Phoenix; California--Mr. James Boykin, Valencia; Dr. L.L. Cavalli-Sforza, Stanford University, Palo Alto; Dr. Michael N. Oxman, V.A. Hospital, San Diego; Delaware--Dr. Roger Rodrique, Wilmington; Hawaii--Dr. Arwin Diwan, University of Hawaii, Honolulu; Dr. Leon Rosen, Pacific Research Center, Honolulu; Don Rubinstein, University of Hawaii, Honolulu; Dr. Gordon Wallace, Pacific Research Station; Illinois--Judith Farquhar, Chicago; Dr. Walter R. Kirschbaum, Chicago; Maryland--Dr. Richard T. Johnson, Johns Hopkins Hospital, Baltimore; Dr. David Lang, University of Maryland, Baltimore; Dr. Guy McKhann, Johns Hopkins University, Baltimore; Dr. Chris Plato, Gerontology Research Center, Baltimore; Dr. Constantine Sakles, University Hospital, Baltimore; Dr. Charles Wisseman, University of Maryland, Baltimore; Dr. K.V. Shah, Johns Hopkins University, Baltimore; Mr. T.C. Rains, National Bureau of Standards, Gaithersburg; Massachusetts--Dr. John Enders, Brookline; Mr. Peter Fetchko, Peabody Museum, Salem; Michigan--Prof. J.V. Neel, University of Michigan, Ann Arbor; Dr. Ernst A. Rodin, Lafayette Clinic, Detroit; Minnesota--Dr. Leonard Kurland, Mayo Clinic, Rochester; Dr. G. Albin Matson, Minneapolis; Nevada--Dr. Warren V. Huber, V.A. Medical Center, Reno; New Jersey--Dr. Karl Maramorosch, Rutgers University, New Brunswick; Dr. Richard Masland, Englewood; New York--Dr. Robert Glasse, Queen's College, Flushing; Dr. Shirley Lindenbaum, The New School, New York; Dr. Ralph D. Peterson, New York Hospital-Cornell Medical Center, New York; Dr. Roger D. Traub, IBM Thomas W. Watson, Yorktown; Ohio--Dr. Richard Feinberg, Kent State University, Kent; Dr. Frank P. Saul, Medical College, Toledo; Dr. Arthur G. Steinberg, Case Western Reserve University; Pennsylvania--Dr. Paul T. Baker, Pennsylvania State University, University Park; Dr. Napoleon Chagnon, Pennsylvania State University, University Park; Drs. Werner and Gertrude Henle, Children's Hospital of Philadelphia, Philadelphia; Rhode Island--Dr. Terrence E. Hays, Rhode Island College, Providence; Dr. John Strom, Rhode Island Hospital, Providence; South Carolina--Dr. Paul M. Hoffman, V.A. Hospital, Charleston; Dr. Albert Sabin, Medical University of South Carolina, Charleston; Texas--Dr. Heather D. Mayor, Baylor University Medical School, Houston; Dr. Steven Wiesenfeld, Southwest Allergy Service, Inc., Midland; Washington--Dr. Ronald DiGiacomo, University of Washington, Seattle; Wisconsin--Dr. G.R. Hartsough, Great Lakes Mink Association, Pittsville; Dr. Richard F. Marsh, University of Wisconsin, Madison; Dr. Gabriel Zu Rhein, University of Wisconsin, Madison.



COOPERATING UNITS: continued

YUGOSLAVIA: Prof. J. Vesenjak-Hirjan, Sveucilistau Zagrebu, Zagreb.

- Sub-Project I: Study of the development patterning of the human nervous system (cybernetics of human development).
- Sub-Project II: Human evolutionary studies in isolated primitive groups.
- Sub-Project III: Studies of isolated Micronesian populations.
- Sub-Project IV: Studies of isolated New Guinea populations.
- Sub-Project V: Studies of Australian Aborigines.
- Sub-Project VI: Studies of isolated New Hebrides and Solomon Islands populations.
- Sub-Project VII: Studies of Central and South American Indians.
- Sub-Project VIII: Developmental, genetic and disease patterns in primitive populations of Asia, Africa, Indonesia, Melanesia, Micronesia, Polynesia and the Arctic.
- Sub-Project IX: Experimental developmental neuropsychiatrics in infantile programming: a empirical approach to the language of information input into the nervous system.
- Sub-Project X: Ciphers and notation for the coding of sensory data for neurological information processing.
- Sub-Project XI: Racial distribution and neuroanatomic variations in the structure of the human brain.
- Sub-Project XII: Studies of high incidence of neurological disease in specific racial and ethnic groups and in primitive or geographic population studies.
  
- Project Description: Neurobiology of Population Isolates: Study of Child Growth and Development, Behavior and Learning, and Disease Patterns in Primitive Cultures (described fully on pages 1-LCNSS/IRP through 27-LCNSS/IRP.
  
- Publications: Listed on pages 41-LCNSS/IRP through 57-LCNSS/IRP.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 00969-17 CNSS
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Chronic CNS Disease Studies: Slow, Latent and Temperate Virus Infections		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PRINCIPAL INVESTIGATORS: D. Carleton Gajdusek, M.D., Chief, LCNSS; and Clarence J. Gibbs, Jr., Ph.D., Deputy Chief, LCNSS		
OTHER: Herbert L. Amyx, D.V.M.; Tomonobu Aoki, M.D.; David M. Asher, M.D.; Sina Baymanyar, M.D.; Maria-Teresa Borrás, Ph.D.; Paul W. Brown, M.D.; Marie-Claude Moreau-Dubois, Ph.D.; Ryo Fukatsu, M.D.; Ralph M. Garruto, Ph.D.; Yasuo Kuroda, Ph.D.; Pyung-Woo Lee, Ph.D.; Maryellen F. Masciangelo, Ph.D.; Maurizio Pocchiarri, M.D.; Robert G. Rohwer, Ph.D.; Andres Salazar, M.D.; Richard T. Yanagihara, M.D.; Francoise Cathala, M.D.; Dimitry Goldgaber.		
COOPERATING UNITS (if any) AUSTRALIA: Dr. Byron A. Kakulas, University of Western Australia, Nedlands; Dr. Chev Kidson, Queensland Institute of Medical Research, Brisbane; Dr. Robert L. Kirk, Australian National University, Canberra; Dr. Ian MacKay, Royal Melbourne Hospital, Melbourne; (continued)		
LAB/BRANCH Laboratory of Central Nervous System Studies, Intramural Research Program		
SECTION		
INSTITUTE AND LOCATION National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20205		
TOTAL MANYEARS: 24	PROFESSIONAL: 14	OTHER: 10
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input checked="" type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Studies elucidate cause and pathogenesis of chronic degenerative CNS disorders with emphasis on MS, ALS, parkinsonism-dementia, Parkinson's, Pick's, and Alzheimer's disease, Huntington's chorea, supranuclear palsy, other presenile dementias, chronic encephalitis with focal epilepsy, muscular dystrophies, chronic schizophrenia, SSPE, PML, dialysis encephalopathy, and intracranial neoplasms. Even familial, apparently hereditary diseases may be slow virus infections. Subacute spongiform virus encephalopathies (kuru and Creutzfeldt-Jakob (CJD) disease of man; scrapie and mink encephalopathy) are caused by unconventional viruses with unique properties posing important theoretical problems to microbiology and molecular biology; a major goal is elucidation of their structure and mechanisms of replication. Transmissible virus dementias are increasingly recognized worldwide causes of death: high incidence foci, transmission by corneal transplant or brain surgery, and occupational hazards from exposure to brain occur. In order to determine the usual mode of infection with the virus, a worldwide epidemiological study of transmissible virus dementia (CJD) cases is underway with special attention to familial clusters of cases and with a quest for possible relationship of scrapie of sheep to the human disease.		

## COOPERATING UNITS: (continued)

AUSTRALIA: Dr. Colin Masters, University of Western Australia, Perth; Dr. Eric Shaw, Red Cross Blood Transfusion Service, Brisbane; Dr. Vincent Zigas, Sunnybank.

AUSTRIA: Prof. F. Seitelberger, University of Vienna, Vienna.

BELGIUM: Dr. A. Lowenthal, L'Institut Bunge, Antwerp.

CANADA: Dr. John H. Deck, Toronto Western Hospital, Toronto; Dr. Joseph Gilbert, University Hospital, London; Dr. Arthur J. Hudson, University Hospital, London; Dr. Andrew Kertez, St. Joseph's Hospital, London; Dr. Theodore Rasmussen, McGill University, Montreal; Dr. N.B. Rewcastle, Banting Institute, Toronto.

CHILE: Dr. Sergio Galvez, Institute de Neurocirugia, Santiago.

CHINA: Prof. Chi-lu Chen, National Taiwan University, Taipei; Dr. C.H. Yen, National Health Administration, Taipei; Dr. Chin-Yun Yii, Kaohsiung Medical College, Kaohsiung.

CUBA: Dr. Segundo Mesa-Castillo, Hospital Psiquiatrico de la Habana, Havana.

CZECHOSLOVAKIA: Dr. Helena Libokova, Slovak Academy of Sciences, Bratislava; Dr. Vlastimil Mayer, Slovak Academy of Science, Bratislava; Dr. Eva Mitrova, Research Institute for Preventative Medicine, Limbova.

EGYPT: Dr. Harry Hoogstraal, Naval Medical Research Unit, Cairo.

ENGLAND: Mrs. Elisabeth Beck, Institute of Psychiatry, London; Dr. M.C. Clark, Agricultural Research Council, Compton; Prof. P.M. Daniel, Royal College of Surgeons, London; Prof. George Dick, Regional Dean's Office, London; Prof. L.W. Duchon, The National Hospital, London; Dr. D.A. Haig, Agriculture Research Council, Compton; Dr. Gordon D. Hunter, Agricultural Research Council, Compton; Prof. W.B. Matthews, University of Oxford, Oxford; Dr. R. Kimberlin, Agricultural Research Council, Compton.

FINLAND: Prof. Nils Oker-Bloom, University of Helsinki, Helsinki.

FRANCE: Dr. Jacques Bert, Centre Hopital et Universite, Marseille; Dr. Francoise Cathala, Hopital de la Salpetriere, Paris; Dr. Henri-Pierre Cathala, Hopital de la Salpetriere, Paris; Dr. Louis Court, Centre de Recherches du Service, Clamart; Dr. Michel Dumas, CHU, Limoges; Prof. A.E. Escourolle, Charles Foix La Salpetriere, Paris; Dr. Henri Gastaut, University of Marseille, Marseille; Dr. Patrick Gourmelon, CRSSA, Clamart; Dr. Raymond Latarjet, Institut du Radium, Paris; Dr. Martin, CHU, Nice; Dr. Francis Rohmer, CHU, Strasbourg; Dr. Michel Samson, CHU, Roen; Dr. Schott, CHU, Lyon; Prof. Tamalet, Hospital de La Timone, Marseille.

## COOPERATING UNITS: (continued)

GERMANY: Dr. Freidrich Deinhardt, Max-van-Pettekoffer Institute, Munich;  
 Dr. Klaus Mannweiler, Henrich-Pette-Institute fur Viroloigie und Immunologie,  
 Hamburg; Dr. W.K. Muller, Country Psychiatric Hospital, Wiesloch;  
 Dr. Volker ter Meulen, Institute fur Virologie, Wurzburg; Dr. Wolfgang Zeman,  
 Lahstein.

GUAM: Dr. Kwang-Ming Chen, NINCDS Research Center, Tamuning;  
 Dr. Leon Concepcion, Guam Memorial Hospital, Agana; Dr. Olivia Cruz, NINCDS  
 Research Center, Tamuning; Jose Torres, NINCDS Research Center, Tamuning;  
 Dr. Yushiro Uebayashi, NINCDS Research Center, Tamuning;  
 Dr. Richard T. Yanagihara, NINCDS Research Center, Tamuning.

ICELAND: Dr. Margret Gudnadottir, University of Iceland, Reykjavik;  
 Dr. P.A. Palsson, University of Iceland, Reykjavik; Dr. G. Petursson, University  
 of Iceland, Reykjavik.

INDONESIA: Dr. Budi Subianto, Public Health Department, Jayapura.

JAPAN: Dr. Fushahiro Ikuta, Brain Research Institute, Niigata;  
 Dr. Kiyotaro Kondo, Brain Research Institute, Niigata; Dr. Reisaku Kono,  
 National Institute of Health, Tokyo; Dr. Yoshigoro Kuroiwa, Kyushu University,  
 Fukuoka; Dr. Takao Makifuchi, Brain Research Institute, Niigata;  
 Dr. Ryoichi Mori, Kyushu University, Fukuoka; Dr. Shigeru Mori, Brain Research  
 Institute, Niigata; Dr. Nobuyuki Murakami, Nagoya University of School of  
 Medicine, Nagoya; Dr. Seiho Nagafuchi, Kyushu University, Fukuoka;  
 Dr. Ikuya Nagata, Nagoya University, Nagoya; Dr. Hiroshi Oda, Kagoshima  
 University, Kagoshima; Dr. Tadao Tsubaki, Tokyo Metropolitan Neurological  
 Hospital, Tokyo; Dr. Yoshio Yase, Wakayama Medical College, Wakayamashi;  
 Dr. Yoshigoro Kuroiwa, Kyushu University, Fukuoka.

KOREA: Dr. Ho Wang Lee, Korea University Medical College, Seoul.

MEXICO: Dr. Julio Sotelo, Institut Nacional de Neurologia, Mexico City.

NETHERLANDS: Dr. Jan ten Brink, University Hospital of Amsterdam, Amsterdam;  
 Dr. Jan van der Noordaa, Laboratorium boor GezondheidsLeer, Amsterdam.

NEW ZEALAND: Dr. R.W. Hornabrook, Wadestown.

PAPUA NEW GUINEA: Dr. Michael Alpers, Institute for Medical Research, Goroka.

PERU: Dr. Luis Palomino, Hospital Santo Toribio, Lima.

POLAND: Dr. P.P. Liberski, Department of Neurology, Lodz;  
 Prof. Dr. Ewa Osetowska, Polish Academy of Sciences, Minsk.

PUERTO RICO: Dr. Victor Mojica, Veterans Administration Center, San Juan.

REPUBLIC OF CHINA: Prof. Chi-lu Chen, National Taiwan University, Taipei;  
 Dr. C.H. Yen, National Health Administration, Taipei; Dr. Chin-Yun Yii,  
 Kaohsinung Medical College, Kaohsiung.

## COOPERATING UNITS: (continued)

SCOTLAND: Dr. Alan G. Dickinson, A.R.C. Animal Breeding Research Organization, Edinburgh; Dr. Hugh Fraser, A.R.C. Animal Breeding Research Organization, Edinburgh; Dr. J.D. MacGregor, Shetland Health Board, Shetland.

SOUTH AFRICA: Dr. J.H.S. Gear, National Institute of Virology, Sandringham.

SPAIN: Dr. J.A. Sanchez-Martin, Instituto de Investigaciones, Madrid; Dr. Alberto Portera-Sanchez, Ciudad Savitaria Primero, Madrid.

SWEDEN: Dr. Erling Norrby, Karolinska Institute, Stockholm; Dr. Arne Svedmyr, Central Bacteriological Laboratory, Stockholm.

SWITZERLAND: Dr. Christoph Bernoulli, Universitsspital, Zurich; Dr. Liana Bolis, World Health Organization, Geneva; Dr. Breget, University of Geneva, Geneva; Dr. B. Ney, University of Geneva, Geneva.

USSR: Dr. Mikhasil Petrovich Chumakov, Institute of Poliomyelitis and Virus Encephalides, Moscow; Dr. Lydia L. Fadeeva, Ulitsa Valtera Ulbrichta, Moscow; Dr. Sophia Janovna Gaidamovich, Ivanovskii Institute of Virology, Moscow; Prof. Vera I. Il'yenko, All-union Research Institute of Influenza, Leningrad; Dr. Prokopii Andrevich Petrov, Yakut Ministry of Public Health, Yakutsk; Dr. Vanda V. Pogodina, The Institute of Poliomyelitis and Virus Encephalitis, Moscow; Dr. Peter Rytik, Biorussian Institute of Epidemiology, Minsk.

UNITED STATES: California--Dr. J. Richard Baringer, V.A. Hospital, San Francisco; Dr. Ashley T. Haase, V.A. Hospital, Palo Alto; Dr. R. Nick Hogan, V.A. Medical Center, San Francisco; Dr. Kenneth P. Johnson, San Francisco; Dr. David E. Kohne, Center for Neurologic Studies, San Diego; Dr. Peter Lampert, University of California, La Jolla; Dr. Edwin H. Lennette, State Department of Health, Berkeley; Dr. Michael N. Oxman, V.A. Hospital, San Diego; Dr. Linus Pauling, Linus Pauling Institute, La Jolla; Dr. Stanley Prusiner, University of California, San Francisco; Dr. Gunther Stent, University of California, Berkeley; Dr. W.W. Tourtellotte, V.A. Hospital, Los Angeles; Dr. Myron Varon, Amyotrophic Lateral Sclerosis Society, Sherman Oaks; Dr. Steven Waxman, Stanford University, Palo Alto; Dr. Leslie P. Weiner, University of Southern California, Los Angeles; Connecticut--Dr. P.N. Bhatt, Yale University, New Haven; Dr. G.D. Hsiung, V.A. Medical Center, West Haven; Dr. Elias and Laura Manuelides, Yale University School of Medicine; New Haven; Hawaii--Dr. Arwin R. Diwan, University of Hawaii, Honolulu; Dr. Scott B. Halstead, University of Hawaii, Honolulu; Dr. Hong-Yi Yang, University of Hawaii, Honolulu; Illinois--Dr. Raymond A. Classen, Presbyterian-St. Luke's Hospital, Chicago; Dr. Raymond Roos, University of Chicago, Chicago; Indiana--Dr. Bernadino Ghetti, Indiana University School of Medicine, Indianapolis; Dr. Morris Pollard, Lobund Laboratory, Notre Dame; Dr. A.N. Siakotos, Indiana University, Indianapolis; Kentucky--Dr. Dan Tynan, V.A. Hospital, Lexington; Louisiana--Dr. William Greer, Gulf South Research Institute, New Iberia; Maryland--Dr. Frederick B. Bank, Johns Hopkins University, Baltimore; Dr. Theodore O. Diener, Agricultural Research Center West, Beltsville; Dr. Richard T. Johnson, Johns Hopkins University, Baltimore;



## COOPERATING UNITS: (continued)

Dr. David Lang, University of Maryland, Baltimore; Mrs. Meta Neumann, Bethesda; Dr. Robert Traub, University of Maryland, Baltimore; Dr. Charles Wissemann, University of Maryland, Baltimore; Dr. K.V. Shah, Johns Hopkins University, Baltimore; Mr. T.C. Rains, National Bureau of Standards, Gaithersburg; Massachusetts--Dr. Amico Bignami, Children's Hospital Medical Center, Boston; Dr. Bernard Fields, Harvard Medical School, Boston; Dr. E. P. Richardson, Jr., Massachusetts General Hospital, Boston; Dr. W.C. Schoene, Peter Bent Brigham Hospital, Boston; Nevada--Dr. Warren V. Huber, V.A. Medical Center, Reno; New York--Dr. Samuel J. Ayl, The National Foundation March of Dimes, White Plains; Dr. Jordi Casals, Mt. Sinai School of Medicine, New York; Dr. Alfred E. Earle, Public Health Research Institute, Otisville; Dr. Teresita S. Elizan, Mt. Sinai School of Medicine, New York; Mr. Ernie Green, New York Public Health Research Institute, Otisville; Dr. Asao Hirano, Montefiore Hospital, Bronx; Dr. John Hotchin, Department of Health, Albany; Dr. J. Moor-Jankowski, New York University Medical Center, New York; Dr. Imaharu Nakano, Montifiore Hospital and Medical Center, New York; Dr. Michael L. Shelanski, New York University Medical Center, New York; Dr. Robert A. Sommerville, New York State Institute for Basic Research in Mental Retardation, Staten Island; Dr. Robert D. Terry, Albert Einstein Medical Center, Bronx; Dr. Roger D. Traub, IBM Thomas B. Watson Research Center, Yorktown Heights; Dr. James D. Watson, Cold Spring Harbor Laboratory, Cold Spring; Ohio--Dr. S.M. Chou, Cleveland Foundation, Cleveland; Dr. Maurice Victor, Metropolitan General Hospital, Cleveland; Pennsylvania--Dr. Milton Alter, Temple University Medical Center, Philadelphia; Dr. Donald Gilden, Wistar Institute, Philadelphia; Dr. Neal Nathanson, University of Pennsylvania School of Medicine, Philadelphia; South Carolina--Dr. Paul M. Hoffman, V.A. Hospital, Charleston; Texas--Dr. Samuel Baron, University of Texas, Galveston; Dr. Steven Wiesenfeld, Southwest Allergy Service, Midland; Virginia--Dr. J.L. Hourrigan, Arlington; Washington--Dr. Ellsworth C. Alvord, Jr., University of Washington, Seattle; Washington, D.C.--Dr. Harold Booker, Veterans Administration Central Office; Col. Dan C. Cavanaugh, Walter Reed Army Institute; Dr. John Kurtzke, V.A. Hospital; Dr. Frederick C. Robbins, National Academy of Science; Dr. Fuller Torrey, St. Elizabeth's Hospital; Wisconsin--Dr. Richard F. Marsh, University of Wisconsin, Madison; Dr. Gabriel Zu Rhein, University of Wisconsin, Madison.

YUGOSLAVIA: Dr. Miha Likar, Mikrobioloski Institut, Ljubljana;  
Prof. J. Vesenjnak-Hirjan, University of Zagreb, Zagreb.

- Sub-Project I: Attempts to isolate, identify and characterize transmissible agents from humans and animals with subacute degenerative diseases of the central nervous system: transmissible hereditary diseases, presenile and senile dementias of the sporadic and familial types and primary sclerosing and demyelinating diseases.
- Sub-Project II: Characterization and pathogenesis of kuru virus.



- Sub-Project III: Characterization and pathogenesis of Creutzfeldt-Jakob disease (transmissible dementia virus).
- Sub-Project IV: Scrapie: studies on the purification, physical and biological characterization and nature of the virus.
- Sub-Project V: In vitro cultivation of the viruses of the subacute spongiform virus encephalopathies in cell cultures.
- Sub-Project VI: Host range of susceptible laboratory animals to the viruses of the subacute spongiform virus encephalopathies.
- Sub-Project VII: Strain variations among the viruses of the subacute spongiform virus encephalopathies.
- Sub-Project VIII: Cell-fusing properties of the viruses of the subacute spongiform virus encephalopathies.
- Sub-Project IX: Resistance to radiation of the viruses of the subacute spongiform virus encephalopathies.
- Sub-Project X: Resistance to disinfectants of the viruses of the subacute spongiform virus encephalopathies.
- Sub-Project XI: Tissue and cell culture techniques used to unmask slow infection of man and animals using brain and viscera biopsy and early autopsy, bone marrow and peripheral leucocyte specimens.
- Sub-Project XII: The syncytium-forming viruses (simian and human foamy viruses).
- Sub-Project XIII: Studies on transformed human brain tissue in vitro and characterization of associated virus.
- Sub Project XIV: Electron microscopic membrane studies of subacute spongiform virus encephalopathies.
- Sub-Project XV: Characterization and identification of new herpes viruses from explant cultures of tissues from subhuman primates.
- Sub-Project XVI: Studies on persistent asymptomatic cytomegalovirus infections of healthy rhesus monkeys.
- Sub-Project XVII: Focal movement disorders in rhesus monkeys following experimental infection with a strain of tick-borne encephalitis virus.

- Sub-Project XVIII: Fluorescent antibody studies on the intracellular localization and identification of virus antigens in vivo and in vitro in tissues from patients with subacute diseases of the central nervous system.
- Sub-Project XIX: Isolation and characterization of adenovirus from the urine of chimpanzees.
- Sub-Project XX: Development of serological and immunological test system for use in the study of slow infections of the central nervous system.
- Sub-Project XXI: Immune responsiveness of multiple sclerosis patients to established viral antigens by detection of specific antibodies in serum and cerebrospinal fluids collected serially during remission and exacerbation.
- Sub-Project XXII: Animal management and intercurrent diseases in subhumans primates on long-term studies of slow infections.
- Sub-Project XXIII: Studies to determine the possible presence of cryptic viral genomes in human brain tissues.
- Sub-Project XXIV: Sequential development of kuru-induced neuropathological lesions in spider monkeys.
- Sub-Project XXV: Studies on the isolation, characterization, identification and pathogenicity of type C viruses from human and animal tissues.
- Sub-Project XXVI: Biochemical studies of the etiology of amyotrophic lateral sclerosis and parkinsonism-dementia.
- Sub-Project XXVII: Study of mitochondrial mutants from scrapie-infected mouse brain cells.
- Sub-Project XXVIII: Isolation and characterization of the etiological agent of Scandinavian nephro-nephritis epidemica.
- Sub-Project XXIX: The pathogenesis of Korean hemorrhagic fever virus and the elucidation of its biological and physical properties.
- Sub-Project XXX: Worldwide seroepidemiological evidence of antibodies in human populations to the virus of Korean hemorrhagic fever.
- Sub-Project XXXI: Development of an enzyme-linked immunoadsorbent (ELISA) test for the diagnosis and epidemiology of cysticercosis-induced epilepsy.

- Sub-Project XXXII: Studies on the cytochemical and morphological properties of neurons cultured in vitro.
- Sub-Project XXXIII: Development of immunological markers for the detection of autoantibodies to neurofilaments in the sera of patients with subacute spongiform encephalopathies.
- Sub-Project XXXIV: Studies to determine the neurophysiological changes of neurons in vitro infected with CJD.
- Sub-Project XXXV: Effects of the subacute spongiform viruses on nerve cells grown in vitro.
- Sub-Project XXXVI: In vivo and in vitro studies to determine the etiology of mysasthenia gravis.
- Sub-Project XXXVII: Neurophysiological study of animals experimentally infected with subacute spongiform virus encephalopathies.
- Project Description: Chronic Central Nervous System Disease Studies (described fully on pages 1-LCNSS/IRP through 27-LCNSS/IRP).

The projects (I through XXXVII) listed herein, as itemized in the Project Reports of previous years, have continued throughout this year and have been expanded, as are reflected in the extensive list of publications and the summary in pages 1-LCNSS/IRP through 27-LCNSS/IRP. Contractural phases of this work are being conducted at: Gulf South Research Institute, New Iberia, Louisiana; and Public Health Research Institute of the City of New York, Inc., Otisville, New York.

Publications: Listed on pages 41-LCNSS/IRP through 57-LCNSS/IRP.

1. Alpers, M., Ono, S., and Gajdusek, D.C. (1980) Progressive slow disappearance of kuru. Abstract No. 93 in Abstracts of the Tenth International Congress on Tropical Medicine and Malaria, Manila, November 9-15, p. 63.
2. Amyx, H.L., Gibbs, C.J. Jr., Gajdusek, D.C., and Greer, W.E. (1981) Absence of vertical transmission of subacute spongiform viral encephalopathies in experimental primates. Proceedings of the Society for Experimental Biology and Medicine 166:4(April), 469-471.
3. Asher, D.M. (1980) Chronic encephalitis. In "Search for the Cause of Multiple Sclerosis and Other Chronic Disease of the Central Nervous System", Proceedings of the First International Symposium of the Hertie Foundation, Frankfurt am Main, September, 1979, A. Boese, editor. Verlag Chemie, Weinheim, pp. 273-279.
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CONTRACTS

Gulf South Research Institute  
New Iberia, Louisiana

Contract #N01-NS-8-09931

\$ 600,000.00

Public Health Research Institute of the City of New York, Inc.  
Otisville, New York

Contract #N01-NS-7-0082

\$ 131,000.00

Litton Bionetics, Inc.  
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Institute of Psychiatry  
London, England

Contract #263-78-C-0049

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# ANNUAL REPORT

October 1, 1980 through September 30, 1981

## Clinical Neurosciences Branch

National Institute of Neurological and Communicative Disorders and Stroke

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## ANNUAL REPORT

October 1, 1980 through September 30, 1981  
Clinical Neurosciences Branch  
National Institute of Neurological and Communicative  
Disorders and Stroke

Susumu Sato, M. D., Acting Chief

### Summary of Program Activity

The Branch activity consists of research and clinical diagnostic service that involves a total of 3.9 man/years (1 professional and 2.9 technical-clerical).

#### I. Clinical Diagnostic Service:

During this reporting period, a total of 1074 electroencephalograms and 206 cerebral evoked response testings (CER) were obtained in patients who are referred to our Branch. As part of their routine clinical investigations or for specific projects from other Branches of our Institute or from other Institutes.

The distribution of these referrals according to the Institute of origin is as follows:

<u>Institute</u>	<u>EEG No.</u>	<u>%</u>	<u>CER No.</u>	<u>%</u>
NINCDS	545	50.7	138	67.0
(OPD)	(234)	(21.7)		
NIMH	178	16.5	8	3.9
NICHD	68	6.3	9	4.4
NCI	60	5.5	12	5.8
NHLBI	38	3.5	3	1.4
NIAID	78	7.2	9	4.4
NIADDK	81	7.5	12	5.8
NEI	5	0.4	6	2.9
MISC	21	1.95	9	4.4
	1074	100.00	206	100.00

There is again a slight increase in the total number of electroencephalograms as compared with that of the previous year. About 50% of the EEG requests came from Institutes other than NINCDS. The service continues to supply useful information for several research projects in our Branch which collaborates closely with other units, especially the Clinical Epilepsy Section of the Experimental Therapeutics Branch.

During the reporting period, as can be seen in the table, a total of 206 cerebral evoked response testings, including visual, brainstem auditory and somatosensory evoked potentials were obtained. Sixty-seven percent of the patients were referred from the NINCDS. The majority of the patients were referred for the test with a question of demyelinating disease.

Miscellaneous categories included the bedside EEG recording in the CCU and electrocortigraphy in the operating room.

The Branch also provides material for the training in Clinical Electroencephalography so that each year one or two Clinical Associates become eligible for the American Board of Qualification's in EEG.

## II. Research Activity:

There are seven projects which are active during this reporting period. All are continued from the previous year. Several collaborative studies are also included.

### a) Clinical

The analysis of clinical seizure patterns in different forms of epilepsy continues to be a main field of interest for this Branch. Toward the end of this reporting period, the Branch installed an EEG machine with Video monitoring system which allows us to critically observe ictal, clinical and EEG patterns simultaneously and which also gives us an opportunity to videotape them for further analysis. This will tremendously increase the chance of electro-clinical correlation.

Electroencephalographic manifestations and cerebral evoked potentials were studied in a variety of neuromuscular disorders (NMD). A total of 101 patients were evaluated retrospectively and prospectively. Their ages ranged between 2 and 75 years, and 48 were females and 53 males. Representative disorders included motor neuron diseases (MND), peripheral neuropathy, congenital myopathy, muscular dystrophy, myasthenia gravis and others. Our results show that only 2 (6%) out of 46 patients with MND had EEG abnormalities, whereas 6 (38%) out of 16 patients with muscular dystrophy showed abnormalities. The main abnormality was background slowing at theta and delta frequency. The frequency of abnormalities in other NMDs were in a range between the above two groups. Results of cerebral evoked potentials, namely prolonged latencies and distorted morphology, seem to support the EEG findings. Our findings corroborate those of the literature and suggest a more generalized systemic involvement in NMD and particularly in the muscular dystrophies.

In collaboration with the NIMH, Clinical Neurologic and Electrophysiologic examinations (EEGs and cerebral evoked potentials) were evaluated in 32 male volunteer college students. Ten had the highest accuracy of smooth pursuit eye tracking (HAT) and 22 the lowest accuracy (LAT). Their ages ranged between 20 and 31 years. The results showed that degree of inaccuracy of smooth pursuit eye tracking correlated significantly with positive neurological findings ( $P < 0.01$ ) and with the presence of an abnormal EEG ( $P < 0.05$ ). All of 4 subjects with EEG abnormalities had a background slowing except for one who also had epileptiform discharges. One subject had an abnormal visual evoked potential, namely prolongation of the major positive peak latency. Three subjects had abnormal brainstem auditory evoked responses: One had unreproducible responses in one ear and two had prolongation and significant side-to-side difference of interpeak latencies. All findings, except one abnormal EEG, were found among the LAT's. These results suggest that impairment of smooth pursuit eye tracking may be a sensitive marker for neurointegrative dysfunction. The absence of gross neurologic impairment in most subjects suggests that individuals with eye tracking impairment may not uniformly have discrete neurologic illness but may have subtle dysfunction that can be detected on neurologic examination and neurophysiologic investigation.

The effect of Enflurane, an anesthetic, on brainstem auditory evoked responses (BAER) has been studied during the course of short abdominal surgical procedures.

The BAER is obtained from Cz with reference to A1 or A2. The testing intensity of stimulating 11Hz clicks is set 70db and the masking noise 40db above the hearing level. Using a Nicolet CA 1000, 2000 clicks are averaged and recorded continuously for 2 to 5 hours.

So far 6 patients have been studied. Enflurane produced a consistent shift in BAER latencies. The waves mostly affected were waves III, IV and V. The observed prolongation in latency was maximum for the highest concentrations of Enflurane and represented a shift of 10 to 13% from the control values. These latency variations followed closely the variations in minimum Alveolar Concentration measured by Medspec II Chemtron Mass Spectrometer.

The latency changes reversed themselves as the concentration decreased. Changes in I-III, III-V and I-V interpeak latencies showed a parallel trend in relation to those of individual peak latencies. The BAER amplitude diminished substantially in two subjects at peak Enflurane concentrations. Our results show that Enflurane modifies the BAER latencies, namely III, IV and V, and support the postulated mechanism that this agent has the primary action site in the brainstem. This project will continue in collaboration with Anesthesiology Section of the Clinical Center.

In collaboration with the Clinical Epilepsy Section, our Branch has been deeply involved in the protocol of positron emission tomography and epilepsy. The Branch provides EEG monitoring before, during and after the PET scanning. The Branch also has been involved deeply in the project of the PET scan in patients with brain tumor where the origin of delta waves is being investigated.

The neuropsychological research program has actively examined cognitive and emotional changes associated with neuropsychiatric diseases, including Huntington's and Alzheimer's Disease. While testing the basic assumption that these disorders are related to structural-functional defects in cortical/subcortical systems, the investigations also examined whether salient dysfunctions relate to an asymmetry of involvement with left or right brain mechanisms.

The general findings confirmed the presence of widespread cognitive and emotional changes in individuals with Huntington's or Alzheimer's Disease. The impairment was appreciable, and emerged in perception and memory, visuospatial integration and in the utility of spatial-directional clues. Moreover, patients with early stage symptoms experienced difficulties in perceiving and encoding sensory messages presented via visual, auditory and tactile channels.

Although it is well known that patients with Huntington's and Alzheimer's Disease exhibit progressive and generalized cognitive decline, especially memory, little is known about the specific patterns of deficits associated with each disorder. To address this question, a series of neuropsychological tests were constructed to sample general intellectual abilities, and were administered to patients and age-matched normal control groups. Both patient groups were found to be globally impaired relative to normal control groups. More significantly, in comparison with Huntington patients, Alzheimer patients were found to be more impaired on tests requiring nonverbal memory and visuo-constructional ability. In contrast, no differences between the neuropsychiatric

patient groups were recorded for linguistic competence. These results were interpreted as reflecting suspect and differential disturbances to subcortical and cortical mechanisms.

In order to assess more precisely the efficiency of brain mechanisms altered by deteriorative neurologic disorders, noninvasive test procedures were designed to study interhemispherical activities. Specifically, tachistoscopic presentations of visual materials to central and lateral visual fields (msec duration), dichotic listening and dihaptic procedures were employed; a matched group of unimpaired, normal subjects was also assessed. Expectedly, the patients in both groups were significantly impaired in test performance, and the younger normal subjects outperformed older individuals. In general, the Huntington patients were characterized by moderate impairment with no consistent evidence of lateralized deficits. In contrast, Alzheimer's patients seemed to exhibit selective and asymmetrical deficits, suggesting impairment of perceptumotor mechanisms commonly assigned to the right hemisphere.

Relatedly, an opportunity was presented to evaluate Parkinsonian Dementia (PD), a variant neuropsychiatric disorder which afflicts a great number of members of the Chamorro race on Guam. A cross-cultured and integrated study was initiated to codify the behavioral and cognitive problems, associated, and eventually, the Branch will compare the observed functional configuration and deterioration process of PD against profiles established for similar disorders on the U. S. mainland. A group of PD patients and matched normal Guamanian subjects were assessed by tasks of sustained attention, memory for visual and auditory memoranda, concept formation and perception.

Marked deficits in memory and attentiveness clearly separated the patients and normal subjects, indicating widespread cognitive impairment. The normal and patient subjects did not differ in skills or ability to formulate and express abstract concepts or principles. This pattern closely paralleled that established for Alzheimer and normal subjects.

A unique case was presented to the staff, and consisted of an individual with agenesis of the corpus callosum: Shapiro's syndrome (with hypothermia). The verbal performance by the patient was average and suggestive of normal cerebral lateralization patterns. However, the patient's performance on tasks of dot detection and localization produced a marked unevenness, in favor of left visual field superiority. The results were interpreted as suggestive of differential processes and roles for the inferior temporal anterior commissure versus the occipital-parietal splenium system which facilitates interhemispherical transfer and processing of visual information.

In a separate study, this patient was examined with several visual recognition procedures in an effort to study the anatomic representation of the vertical visual field midline, and projection to the occipital regions in both hemispheres. In viewing chimeric random patterns, normal subjects perceived visual material from one side of the central field or the other independently, whereas the opposite was true for the acallosal patient. Qualitatively, the errors were different and suggest that bilateral representation of the vertical visual field midline exists, where in normal individuals, functional information may be suppressed via the corpus callosum.

Estimates of intellectual and memory functions and personality traits for



neuropsychiatric patients will be correlated with biochemical and radiographic parameters, and indices of dementia. In addition, electrophysiologic correlates are being studied to better understand how demented patients perceive, learn and respond to a variety of sensory impressions. These data will be invaluable to advance knowledge regarding the general breakdown of neurosensory and cognitive systems produced by Alzheimer's and other psychiatric processes with neurologic disorders.

b) Experimental Research:

Upon completion of kindling technique in rats, the effect of wakefulness and sleep on kindling process has been studied. It has been found that kindled seizures developed much faster and after-discharges were sustained longer during REM sleep than during wakefulness. Experiment on the kindling process during non-REM sleep is in progress. Our observation casts a serious doubt on the notion that kindling process may be a model of learning process. We also are in the process of analyzing neurochemical substances such as cyclic nucleotides AMP and GMP, GABA, ATP, glutamate, phosphocreatinine and so forth in regional brain areas. The goals for this study are to determine the relationship of cerebral metabolism as it relates to the onset, propagation and termination of the seizure process.

Other activities, Honors etc.:

During this reporting period, the Acting Chief has been appointed as chairman of the Infectious Diseases Committee to the American EEG Society and has been charged to develop guidelines for handling patients with infectious disease and cleaning EEG equipment after use. The Chief also served as Associate Examiner for the American Board of Qualification in Electroencephalography.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 00200-27-CN
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less) Cognitive and Emotional Profile of Neuropsychiatric Disorders.  Former Title: Involuntary Movements		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:   OTHER:	P. Fedio A. Martin P. Brouwers C. Cox J. Bravo	Psychologist CN NINCDS Psychologist CN NINCDS Psychologist CN NINCDS Psychologist CN NINCDS Psychologist CN NINCDS
COOPERATING UNITS (if any)  Experimental Therapeutics Branch, NINCDS		
LAB/BRANCH Clinical Neurosciences, IRP		
SECTION Office of the Chief		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.7	PROFESSIONAL: 1.2	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  A neuropsychological profile of <u>dementia</u> and associated <u>emotional</u> and <u>cognitive</u> changes was drafted for individuals with <u>Alzheimer's Disease</u> , <u>Huntington's Disease</u> and those classified as ' <u>at risk</u> ' for <u>Huntington's Disease</u> . The evaluations extended into <u>memory</u> , <u>learning</u> and <u>perceptual</u> areas, and included <u>personality</u> and <u>emotional</u> measures, utilizing standard and experimental tasks, also establishing normative references for the aging processes. These behavioral data will be collated with biochemical and neuroradiometric measures, and independent indicators of deterioration and dementia will be developed.		

## Project Description:

### Objectives:

To develop a comprehensive neuropsychological study of the aging process, and to evaluate specifically, individuals presented with neuropsychiatric disorder as Alzheimer's or Huntington's Disease. The investigation was designed to 1) evaluate attentional, perceptual, memory and behavioral/emotional defects associated with neuropsychiatric disorders; 2) provide an objective assessment of cognitive and behavioral parameters reflecting changes in senescence.

### Methods Employed:

An integrated neuropsychological test battery, comprised of standard and experimental procedures, was developed and included personality-affective inventories. In cognitive areas, standard psychometric tests of memory and intelligence were selected, supplemented by laboratory procedures to codify perception, attention, spatial orientation, learning/memory and adaptive behavior. In addition, specialized techniques, creating non-invasive and functional correlates for interhemispherical competition also formed an integral part of the evaluative procedures.

### Major Findings:

The findings were consonant with emerging neurobehavioral research, describing pervasive and severe cognitive and emotive impairment in individuals with Alzheimer's or Huntington's Disease.

Alzheimer patients were compared to normal controls, matched for age and level of education, on a variety of tests of language functions, with particular attention to verbal fluency and production, and comprehension. In addition, two experimental procedures were designed to explore more subtle properties of meaning: 1) the ability to make judgments about the affective loading of single words and 2) the ability to match a printed word with an abstract pictorial representation, requiring the appreciation of both denotative and connotative meaning and symbolic representation.

The results indicated that, although performing at significantly lower levels than normal individuals with similar educational experiences, the Alzheimer patients also performed within the normal range on a few, selected subtests. Marked impairment was found on tests of comprehension, object naming, and fluency. In addition, the patients with dementia performed worse than normals on the tests of meaning, except when judgments requiring the appreciation of emotional significance were required.

Qualitative analysis of error patterns revealed a specific breakdown in language processes. This was characterized by a loss of knowledge about specific object attributes whereas knowledge for broad categorical information was preserved. This defect, coupled with spatial imperception, impairs daily activities of demented patients, especially in novel or unstructured situations. In general, it was found that the patient group demonstrated marked deficits in learning

and memory under almost all conditions. However, no evidence of any qualitative differences in memory processes were noted between demented and aged-matched subjects.

Analysis of the type of errors which Alzheimer patients made on recognition tasks did, however, uncover a specific loss. In a special memory (recognition) paradigm, the subjects had to identify a target word which originally appeared on a list of words they had just learned, and which was embedded among three distractor words: a semantically, a phonemically, and an unrelated, word. It was found that the overwhelming majority of patients incorrectly selected the semantic distractor word, that is, retrieving a word from the category or concept containing the original target word. This finding was interpreted as suggesting a breakdown in the ability of demented patients to distinguish between items within the same semantic or conceptual category, a finding consistent with our studies of language dysfunction in this neuropsychiatric population.

In a separate investigation, interhemispherical tasks utilized special visual (lateral fields), auditory (dichotic), and tactile (di-haptic) techniques with patients presenting the early stages of Alzheimer's and Huntington's dementia. It was found that both groups showed pronounced deficits when compared to age-matched controls. However, the specific patterns of deficits were different for the patient groups, and the differential profile reflects underlying differences in neuropathology: degeneration of the frontal-striatal system in the case of Huntington's disease and dysfunction of the mesial temporal structures and the cortical temporal - parietal association regions for Alzheimer's disease.

#### Significance to Biomedical Research and the Program of the Institute:

In view of the cognitive and emotional disabilities associated with neuropsychiatric disorders of dementia as Alzheimer's and Huntington's Disease, the project established an empirical approach to identify neuropsychological deficits which are both common and distinct or orthogonal to each disease process. These observations will be cross-referenced to neuroradiographic and biochemical data in an effort to develop a better understanding of the early and deteriorative course in functional and neurologic sectors, and to evaluate these changes in the context of the normal aging process.

Proposed Course of the Project: The major study of the Huntington's subjects has been completed but new neuropsychological procedures are being developed to monitor progressive changes for patients, and those classified as 'at-risk', time-referenced to the disease. In addition, a large series of Alzheimer's patients have recently been studied and the investigation should be completed within the next several months. The proposal intends to establish a neuropsychological profile of dementia, including specific alterations in mood and cognition that are distinctly and uniquely associated with Alzheimer's or Huntington's Disease, and to evaluate selective radiographic and neuropathological changes.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE  
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF  
HEALTH AND HUMAN SERVICES  
PUBLIC HEALTH SERVICE  
NOTICE OF  
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 NS 01245-16-CN

PERIOD COVERED

October 1, 1980 to September 30, 1981

TITLE OF PROJECT (80 characters or less)

EEG Learning Correlates Using Scalp and Intracranial Depth Electrodes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER  
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI: P. Fedio  
P. Brouwers  
OTHER: M. Buchsbaum

Psychologist CN NINCDS  
Psychologist CN NINCDS  
Research Medical Officer  
BPB NIMH

A. Martin  
C. Cox

Psychologist CN NINCDS  
Psychologist CN NINCDS

COOPERATING UNITS (if any)

Biological Psychiatry Branch, NIMH

LAB/BRANCH

Clinical Neurosciences, IRP

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

0.4

PROFESSIONAL:

0.2

OTHER:

0.2

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS

☐ (b) HUMAN TISSUES

☐ (c) NEITHER

☐ (a1) MINORS ☒ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Information processing by the human brain was monitored and quantified by averaged evoked response techniques. The electrographic activity was recorded from left and right brain regions during perception in normal subjects, and was compared with that of patients with neuropsychiatric disorders (Alzheimer's). Suspect electrographic disturbances in brain-behavior relations in psychiatric patients were also evaluated, relating left brain dysfunction to ideational disorders, and right brain activity to maladaptive emotional reactions.



## Project Description:

### Objectives:

Evoked response measures were included as part of an integrated investigation of altered functions in patients with neuropsychiatric disorders in an attempt to assess brain integrity and modes of perception, interpretation and learning.

### Methods Employed:

A series of attentional tasks were utilized with subjects while electroencephalographic (EEG) activity was recorded and averaged as evoked response patterns from scalp electrodes positioned symmetrically at temporal-parietal regions of the left and right hemispheres. Included for study were neurosurgical patients who had undergone unilateral removal of the temporal lobe and matched normal subjects, and a group of patients with dementia (Alzheimer's Disease).

### Major Findings:

All electrographic test runs were conducted off-line, and the evoked potential data are currently being processed. The study with the temporal epileptic patients is in progress. Preliminary observations with Alzheimer patients revealed anomalies in EEG frequency/amplitude, corresponding to attentional and perceptual deficits in behavior.

### Significance to Biomedical Research and the Program of the Institute:

Behavioral data available from epileptic patients following unilateral temporal lobectomy revealed significant patterns of impairment which are specific to the laterality of surgery. The techniques employed in this project afford a more precise method for outlining cortical and sub-cortical systems which mediate learning and memory in the human brain. The research also extends physiologic and behavioral data for the comparison of neurologic and psychiatric patients in order to better understand the possible linkage between brain dysfunctioning and psychiatric disorders.

Proposed Course of the Project: A computer has been acquired and programs will be developed to provide real time and off-line analysis of data. Specialized neuropsychological tasks will be developed and applied in the study of patients with neuropsychiatric disorders.

Publications: None



SMITHSONIAN SCIENCE INFORMATION EXCHANGE  
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF  
HEALTH AND HUMAN SERVICES  
PUBLIC HEALTH SERVICE  
NOTICE OF  
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 NS 01424-15-CN

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Response Modulation by the Limbic System in Man: Neuropsychological and Physiological Changes

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Fedio	Psychologist	CN	NINCDS
	A. Martin	Psychologist	CN	NINCDS
	P. Brouwers	Psychologist	CN	NINCDS
OTHER:	C. Cox	Psychologist	CN	NINCDS
	J. Bravo	Psychologist	CN	NINCDS

COOPERATING UNITS (if any)

None

LAB/BRANCH

Clinical Neurosciences, IRP

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.1

PROFESSIONAL:

0.6

OTHER:

0.5

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS

☐ (b) HUMAN TISSUES

☐ (c) NEITHER

☐ (a1) MINORS ☒ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

Emotional and cognitive characteristics are studied in epileptic patients with unilateral left or right temporal lobe injury. Temporal epileptic patients are compared with matched normal subjects and patients with other neurologic disorders. The epileptic patients judge and learn information conveying different emotional states, while behavioral and physiological events are recorded. The research examines the role of the temporal lobe in establishing specific limbic associations between the left and right hemispheres in regulating cognitive functions and emotional experiences in man.

## Project Description:

### Objectives:

1. To identify and chart cognitive profiles of epileptics who have undergone unilateral temporal lobectomy for the relief of intractable seizures; to evaluate relationships between onset and length of disorder, and seizure patterns and frequency in relation to behavioral parameters and the therapeutic effects of resections.
2. To evaluate the role of the temporal lobe in guiding and regulating 'emotional perception and learning', and to examine how affective changes and maladaptive behaviors are associated with epilepsy.

### Methods Employed:

Specialized neuropsychological techniques and procedures are being designed and evaluated, and will include tests to identify attentional, perceptual, memory and communicative processes, and the role of language encoding to facilitate recall. The formation and appropriateness of adaptive emotional strategies utilized by brain-injured patients will also be examined.

### Major Findings:

The study is being redesigned and has not yielded patient data. New procedures and techniques are being developed and will be studied in a pilot project with selected patients and control subjects.

### Significance to Biomedical Research and the Program of the Institute:

By identifying specific behavioral sequelae of temporal lobe epilepsy, these observations extend neuroanatomical correlations between brain mechanisms and emotional processes in man. The results may be examined in a hypothesis of enhanced versus diminished sensory-limbic associations. The interpretation regarding the effects of temporal lobe epilepsy in human subjects is consistent with extensive animal experimentation on sensory-limbic disconnections. The findings quantitatively support an asymmetry of emotional and cognitive processing by the right and left hemisphere of man.

Proposed Course of the Project: Develop new behavioral and physiologic procedures, and evaluate additional psychiatric and neurologic groups (nontemporal epileptics).

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE  
PROJECT NUMBER (Do NOT use this space)

U.S. DEPARTMENT OF  
HEALTH AND HUMAN SERVICES  
PUBLIC HEALTH SERVICE  
NOTICE OF  
INTRAMURAL RESEARCH PROJECT

PROJECT NUMBER

Z01 NS 01658-14-CN

PERIOD COVERED

October 1, 1980 through September 30, 1981

TITLE OF PROJECT (80 characters or less)

Hemispheric Development and Specialization of the Intellectual Functions

NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER  
PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	P. Fedio	Psychologist	CN	NINCDS
Other:	C. Cox	Psychologist	CN	NINCDS
	P. Brouwers	Psychologist	CN	NINCDS
	A. Martin	Psychologist	CN	NINCDS
	J. Bravo	Psychologist	CN	NINCDS

COOPERATING UNITS (if any)

None

LAB/BRANCH

Clinical Neurosciences

SECTION

Office of the Chief

INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Maryland 20205

TOTAL MANYEARS:

1.6

PROFESSIONAL:

0.6

OTHER:

1.0

CHECK APPROPRIATE BOX(ES)

☒ (a) HUMAN SUBJECTS

☐ (b) HUMAN TISSUES

☐ (c) NEITHER

☐ (a1) MINORS ☒ (a2) INTERVIEWS

SUMMARY OF WORK (200 words or less - underline keywords)

The disabling effects of brain damage in man were evaluated by a broad range of neuropsychological tests evaluating cognitive functions. Changes in the intellectual behavior of neurologically impaired individuals were evaluated before and after brain surgery, and during electrical stimulation of the cortical surface and subcortical depths.

## Project Description:

### Objectives:

1. Outline cortical and subcortical mechanisms which mediate cognitive functions and code information to be held for immediate (short-term) or delayed (long-term) memory. Compare the effects of injury to different brain regions and develop an integrated model of the human brain for communicative skills and memory processes.
2. Examine the role of temporal lobe mechanisms in guiding visual behavior under altered or distorted perceptual conditions.

### Methods Employed:

The laterality and location of cortical and subcortical zones instrumental in basic cognitive functions were mapped by electrical stimulation and pharmacologic procedures. A verbal and nonverbal test, each with a perceptual and memory command were employed, and utilized photographs of commonplace objects or patterns, with instructions to name or discriminate, and to remember each stimulus object or design.

### Major Findings:

Brief electrical stimulation within the left temporal parietal cortex produced transient dysphasia, speech errors and an independent retrograde memory loss for verbal memoranda. In contrast, comparable electrical stimulation of homologous sites within the right cortex did not interfere with speech or verbal behavior. Instead, the patients experienced difficulty in matching and remembering complex visual patterns. Stimulation of sites situated at the foot of the third frontal gyrus (Broca's area) induced fewer naming errors, while stimulation of the anterior left temporal cortex rarely elicited difficulty in naming. It should also be noted that cortical speech mapping did not elicit reports of experiential or personal memories.

Stimulation of right brain regions also produced a functional distinction between the anterior and posterior regions. As with naming errors and left brain stimulation, the occurrence of visual perceptual errors was coincident with stimulation of the right posterior, but not the right anterior cortical temporal surface. Similarly, errors of omission were the most prevalent on the pattern discrimination tasks.

As part of a preoperative diagnostic evaluation, the patients underwent intracarotid Amytal injection (Wada procedure) to establish laterality of speech mechanisms, and to provide an assessment of memory capabilities, particularly in cases with suspect bi-temporal involvement. The patients received 125mg of Sodium Amytal during which time he or she performed a serial task involving both object naming and memory. During the course of such examination, several individuals were classified as being right-hemisphere dominant; that is, owing to early injury to cortical language zones in the left hemisphere, verbal

functions were readapted to the intact, right hemisphere.

A comparison of performance during and following Amytal injection for individuals designated as left or right brain speech cases revealed that irrigation of the hemisphere dominant for language (left or right) produced a period of object-naming difficulties (dysnomia) and a very long-lasting disruption in verbal memory. In contrast, drug anesthetization of the minor hemisphere for both groups produced no naming errors and significantly less impact on verbal memory.

In comparison with the left dominant group, the right hemisphere speech patients were slower to resume and less accurate to maintain name-recall responses following deactivation of the dominant (right hemisphere). Moreover, there were qualitative differences in the character of dysphasic errors committed by both groups. These preliminary findings suggest that when language mechanisms are realigned to the right cerebral hemisphere, linguistic competence and efficiency, including memory, may be reduced.

Significance to Biomedical Research and the Program of the Institute: These investigations contribute to the basic understanding of the development and organization of structural-functional relations in the brain of man. This research advances clinical knowledge of the relationships between brain dysfunctions and amnesia, dysphasia, dyslexia and specific behavioral or adaptive responses.

Proposed Course of the Project: Tests and procedures are being designed to examine adaptive strategies used by neurologic patients to compensate for communicative, visuomotor and language disorders. Visual and auditory tasks will be developed to further delineate immediate and long-term memory disorders in patients with cortical and subcortical lesions.

Publications: Fedio, P. and Van Buren, J. Thalamo-cortical mediation of perception and memory in man. Adv. Physiol. Sci., 17: 305-312, 1980.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02269-05 CN										
PERIOD COVERED <b>October 1, 1980 through September 30, 1981</b>												
TITLE OF PROJECT (80 characters or less)  <b>Visual Evoked Potentials in Clinical Neurology and Neuro-Ophthalmology</b>												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">P. I.:</td> <td style="width: 33%;">S. Sato, M. D.</td> <td style="width: 33%;">Acting Chief</td> <td style="width: 10%;">CN</td> <td style="width: 10%;">NINCDS</td> </tr> <tr> <td>OTHER:</td> <td>J. Chassy</td> <td>EEG Technologist</td> <td>CN</td> <td>NINCDS</td> </tr> </table>			P. I.:	S. Sato, M. D.	Acting Chief	CN	NINCDS	OTHER:	J. Chassy	EEG Technologist	CN	NINCDS
P. I.:	S. Sato, M. D.	Acting Chief	CN	NINCDS								
OTHER:	J. Chassy	EEG Technologist	CN	NINCDS								
COOPERATING UNITS (if any)  <b>Clinical Epilepsy Section, ETB, NINCDS</b>												
LAB/BRANCH <b>Clinical Neurosciences, IRP</b>												
SECTION <b>Clinical Neurophysiology</b>												
INSTITUTE AND LOCATION <b>NINCDS, NIH, BETHESDA, MD 20205</b>												
TOTAL MANYEARS: <div style="text-align: center;">0.5</div>	PROFESSIONAL: <div style="text-align: center;">0.2</div>	OTHER: <div style="text-align: center;">0.3</div>										
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS												
SUMMARY OF WORK (200 words or less - underline keywords)  <p>           An analysis of the morphology, amplitude and latency of <u>visual evoked potentials</u> to photic flashes and reversing checkerboard pattern being conducted. Normative data have been collected from normal individuals, predominantly of 20-39 years. Visual evoked responses also have been examined in patients with various neurological disorders. Prolonged latencies of the major positive peak have been noted in patients with multiple sclerosis and neurological disorders.         </p>												

## Project Description:

### Objectives:

The practicability and diagnostic value of this test has been and continues to be evaluated in Clinical Neurology. The examination of normal individuals and patients with various neurological disorders will aid our understanding of the mechanism of the VEP.

### Methods Employed:

Initially, photic flash was predominantly used but soon it was found that results were inconsistent intra-as well as inter-individually. Therefore, the mode of stimulation was changed to reversing checker-board pattern. However, flash stimulation has been used in patients who are uncooperative or unconscious. Evoked responses are averaged by the Nicolet CA 1000, analyzed on line and recorded with X-Y plotter. The routine electrode montage consists of O1, O2 and OZ referred to FZ.

### Major Findings:

None. However, we are in the process of designing a study in which pattern shifting and flash visual evoked potentials will be compared in normal subjects. The first step is to obtain normative data with both types of stimulations in the same normal individual. Then we will proceed with applying the test to various neurological conditions.

### Significance to Biomedical Research and the Program of the Institute:

Evoked potentials are useful in the detection of occult lesions in the nervous system, and in establishing the presence of visual function in patients with extensive and neurologic disease. The exclusive and varied patient population in NINCDS would provide an opportunity to study evoked potentials in a variety of disease entities.

### Proposed Course of Projects:

This project will continue.

### Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02431-02 CN										
PERIOD COVERED October 1, 1980 through September 30, 1981												
TITLE OF PROJECT (80 characters or less)  Experimental Epilepsy: Seizures Produced by Kindling in Rat												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">S. Sato, M. D.</td> <td style="width: 20%;">Acting Chief</td> <td style="width: 10%;">CN</td> <td style="width: 20%;">NINCDS</td> </tr> <tr> <td>OTHER:</td> <td>Y. Mohri, M. D.</td> <td>Visiting Scientist</td> <td>CN</td> <td>NINCDS</td> </tr> </table>			PI:	S. Sato, M. D.	Acting Chief	CN	NINCDS	OTHER:	Y. Mohri, M. D.	Visiting Scientist	CN	NINCDS
PI:	S. Sato, M. D.	Acting Chief	CN	NINCDS								
OTHER:	Y. Mohri, M. D.	Visiting Scientist	CN	NINCDS								
COOPERATING UNITS (if any)  Clinical Epilepsy Section, ETB, NINCDS												
LAB/BRANCH Clinical Neurosciences, IRP												
SECTION Clinical Neurophysiology												
INSTITUTE AND LOCATION NIH, NIH, Bethesda, Maryland 20205												
TOTAL MANYEARS: 0.8	PROFESSIONAL: 0.6	OTHER: 0.2										
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS												
SUMMARY OF WORK (200 words or less - underline keywords)  Seizures produced by <u>chronic stimulation</u> (Kindling) are a good model for <u>human epilepsy</u> . In rat, seizures are produced by daily electrical stimulation of amygdaloid complex and other central nervous system sites. In this project, Kindling of the various sites of the central nervous system, interictal epileptiform discharges and their propagation, and effects of sleep-wake cycles and maturation on the epileptiform discharges are being investigated.												

Projection Description:Objectives:

To increase the understanding of mechanism of epileptic seizures. Kindled seizures produced by daily electrical stimulation in rats are being studied from the neurophysiological point of view.

Methods Employed:

Male Sprague-Dawley rats weighing between 350 and 400 grams are anesthetized by intraperitoneal Pentobarbital and a bipolar stainless steel electrode is stereotaxically implanted in the amygdala bilaterally. Screw electrodes are also secured in the skull to monitor the cortical EEGs. The stimulation consists of bipolar pulses of 100 to 200  $\mu$ A, 60Hz and 1 second duration, and is given once a day. The EEG and behavioral manifestations are observed before, during and after stimulation. The rats are stimulated until spontaneous seizures are observed. Long term recording of the EEGs also are made periodically to observe spontaneous interictal discharges and their propagation. At the completion of the experiment, the rats are sacrificed and perfused with formaline and ferrocyanide for histological confirmation of the electrode position.

Major Findings:

We examined the effects of wakefulness and sleep on the amygdala kindling process in rats. The number of daily electrical stimulations (100-200  $\mu$ A) required to fully kindle the animals ranged from 10 to 38 (mean 7.1) during wakefulness (N=12) and from 3 to 12 (mean 6.7) during REM Sleep (N=7). The difference in the kindling rate between wakefulness and REM Sleep was significant ( $P < 0.01$ ). The duration of after discharges was also longer during REM Sleep than during wakefulness. Kindled seizures developed much faster and after-discharges were sustained longer during REM Sleep than during wakefulness. The experiment on the process of being kindled during non-REM Sleep is underway.

Significance to Biomedical Research and Program of the Institute:

The Kindling model of epilepsy is unique in that chronic stimulation at the same intensity (initially subthreshold) eventually produces epileptic seizures. This model is analogous to chronic human epilepsy. Further understanding of kindled seizures in rats will in turn elucidation of the mechanism of the human epilepsy.

Proposed Course of Project: This project will continue.

Publications: The abstract titled "Kindling during wakefulness and sleep in rats" was submitted to the Epilepsy International Symposium in Kyoto, Japan.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02432-02 CN										
PERIOD COVERED <p style="text-align: center;">October 1, 1980 through September 30, 1981</p>												
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Brainstem Auditory Evoked Potentials in Clinical Neurology</p>												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI:</td> <td style="width: 33%;">S. Sato, M. D.</td> <td style="width: 33%;">Acting Chief</td> <td style="width: 10%;">CN</td> <td style="width: 10%;">NINCDS</td> </tr> <tr> <td>OTHER:</td> <td>J. Chassy</td> <td>EEG Technologist</td> <td>CN</td> <td>NINCDS</td> </tr> </table>			PI:	S. Sato, M. D.	Acting Chief	CN	NINCDS	OTHER:	J. Chassy	EEG Technologist	CN	NINCDS
PI:	S. Sato, M. D.	Acting Chief	CN	NINCDS								
OTHER:	J. Chassy	EEG Technologist	CN	NINCDS								
COOPERATING UNITS (if any)  <p style="text-align: center;">Clinical Epilepsy Section, ETB, NINCDS</p>												
LAB/BRANCH <p style="text-align: center;">Clinical Neurosciences, IRP</p>												
SECTION <p style="text-align: center;">Clinical Neurophysiology</p>												
INSTITUTE AND LOCATION <p style="text-align: center;">NINCDS, NIH, Bethesda, Maryland 20205</p>												
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:										
0.5	0.2	0.3										
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS												
SUMMARY OF WORK (200 words or less - underline keywords)  <p> <u>Analysis of the morphology, amplitude and latency of brainstem auditory evoked responses to clicks is being conducted.</u> Normative data have been collected from normal subjects, predominantly of 20-29 years. The test has been carried out in patients with various neurological disorders. Prolonged latencies and distortion of morphology have been observed in patients with Multiple Sclerosis and Spinocerebellar degeneration. The effect of pharmacological agent on the evoked responses also is studied.         </p>												



## Project Description:

### Objectives:

The practicality and diagnostic value of this test has been and continues to be evaluated in Clinical Neurology. The examination in normal subjects and patients with various neurological disorders will help our understanding of the mechanism of brainstem auditory evoked potentials.

### Methods Employed:

Clicks of 10-11Hz, 70db above threshold generalized Nicolet 1007 and delivered to the testing ear through a head phone and masking noise is delivered to the contralateral ear at 40db above the threshold. The brainstem auditory responses are recorded from CZ referred to A1 and A2, and averaged 1000 to 2000 times by the Nicolet CA 1000. The final record is made by an X-Y plotter.

### Major Findings:

In collaboration with the Anesthesiology Section, CC, the effect of Enflurane on brainstem auditory evoked responses (BAER) has been studied. General Anesthesia in 6 patients with normal hearing undergoing abdominal surgery was induced with thiopental and maintained with muscle relaxant and Enflurane for the duration of 2 to 5 hours. Enflurane concentration was determined using a Medspec II Chemtron mass spectrometer which provided alveolar (i.e. blood) concentration. The brainstem auditory evoked potential was obtained from CZ with reference to A1 or A2, namely right or left ear lobes. When the right ear lobe was stimulated the responses were obtained from the channel connecting CA and A2. The ground electrode was placed at FZ position. Before testing, hearing threshold to 11Hz clicks which were generated by Nicolet noise masking unit 10070A was determined on each patient. The testing intensity of stimulating clicks was set 70db above the hearing level and masking noise to the contralateral ear 40db above the hearing level. Using Nicolet CA 1000, 2000 clicks were averaged with analysis time of 10 milliseconds and an input gain of  $\pm 10$  microvolt setting. The responses were immediately transferred on the paper with a Nicolet XY Plotter and a positive peak latencies at CZ position were measured with cursors on the averager. It took about five minutes to obtain one set of responses and the same procedure was repeated as soon as the previous one was secured. The EEG signal was amplified by the Nicolet preamplifier ten thousand times and then led to the averager's amplifiers with again setting of  $\pm 10$  microvolt. The day before the testing was done on all patients had the brainstem auditory evoked potential at the laboratory and all had normal responses. The ear to be stimulated during the testing was selected based upon clarity of responses obtained during pretesting. Enflurane administered at clinical concentrations (0.5, 1, 1.5, 2, 2.5 alveolar concentration)

created constant shift in BAER latencies. The waves mostly affected were waves III-IV and V. The observed prolongation in latency was maximum for the highest concentrations (i.e. deepest level of anesthesia) and represented a shift of 10 to 13% of the control values. These latency variations followed closely the variations in alveolar concentrations and reversed themselves as concentrations were decreased. I-III, III-V and I-IV interpeak latencies showed a parallel trend related to individual peak latency changes. BAER amplitude diminished substantially in two subjects at peak Enflurane concentrations for waves III and IV. In addition, in a few occasions at high anesthetic concentrations, the BAER wave form lost its sharpness (without loss of amplitude) and some waves were difficult to read. Our study shows that Enflurane modifies BAER latencies, specially Waves II, IV and V. This finding can be interpreted in the light of the postulated mechanism of action that this agent acts on the reticular activating system of the brainstem.

#### Significance to Biomedical Research and the Program of the Institute:

Evoked potentials are useful in the detection of occult lesions in the nervous system, particularly in the brainstem/auditory pathway. The extensive and varied patient population in NINCDS would provide an opportunity to study evoked potentials in a variety of disease entities.

#### Proposed Course of Project:

This project will continue.

#### Publications:

Dubois, M., Sato, S., Chassy, J., and Macnamara, T.: Effect of Enflurane on Brainstem Auditory Evoked Potentials (BAER) (Abstract) 35th Annual Meeting of the American Electroencephalographic Society, June 11-13, 1981. Chicago, Illinois.





# ANNUAL REPORT

October 1, 1980 through September 30, 1981  
Developmental and Metabolic Neurology Branch  
National Institute of Neurological and Communicative Disorders and Stroke

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## ANNUAL REPORT

October 1, 1980 through September 30, 1981  
Developmental and Metabolic Neurology Branch  
National Institute of Neurological and Communicative Disorders and Stroke  
Roscoe O. Brady, Chief

Progress in basic and clinical investigations concerning the etiology and treatment of neurological diseases has continued in a highly satisfactory fashion. The principal activities of the Branch relate to the following topics. 1. The metabolism of complex lipids and mucopolysaccharides in normal and pathological states and the treatment of patients with hereditary metabolic disorders. 2. The role of glycolipids and glycoproteins as biotransducers of environmental signals. 3. The function of myelin glycoproteins in the developing nervous system and in the pathogenesis of demyelinating diseases. 4. The relation of cell surface enzymes to intercellular communication and excitability. 5. Enzymes in normal and neoplastic tissue that catalyze the cleavage of glucose from amygdalin (Laetrile). The accomplishments in these endeavors during FY 81 are described.

### I. HEREDITARY METABOLIC DISORDERS

#### A. Enzyme Replacement Therapy for Lipid Storage Diseases

We have continued our investigation of the effects of administration of purified enzyme to patients with Gaucher's disease. The two initial recipients who have now been treated over a period of three and one-half years continue to be in good health and have not required splenectomy for thrombocytopenia that is a frequent manifestation of this disorder. The general health of these boys is improved; their growth patterns are more in line with their age; and there appears to have been an arrest of the progression of the hepatosplenomegaly characteristic of this disease. Because of these encouraging results, we have increased the number of patients in this trial to ten. Results obtained in an independent study of our enzyme preparation by investigators at the Clinical Research Centre in Harrow, England, confirm the arrest of the spleen and liver enlargement in Gaucher patients. Their data will be presented at a meeting on metabolic diseases in New York in July.

#### B. Specific Targeting of Exogenous Enzymes

The effect of enzyme replacement in older patients with Gaucher's disease has been less consistent. A major obstacle encountered is that most of the injected placental enzyme is taken up by hepatocytes in the liver rather than by reticuloendothelial cells where the offending lipid is stored. We are attempting to overcome this obstacle in the following fashion. 1. We have found that modifying the enzyme so that the terminal sugar of its oligosaccharide chains in mannose significantly increases the uptake of enzyme by Kupffer cells in animals. We are currently scaling-up procedures to obtain a sufficient quantity of this target-cell directed enzyme for clinical trials. 2. We have also begun to link mannose residues covalently to the native enzyme by two specific techniques; one was developed here; the second is a collaborative study with investigators at the Merck Institute for Research in Rahway, N.J. If the enzyme

modified by either of these procedures is effectively delivered to reticuloendothelial cells, we propose to prepare larger quantities of it for clinical trials.

#### C. Development of Patient-derived Macrophage Cultures

We have recently developed a tissue culture system using the patient's own macrophages in order to assess the effect of stored glucocerebroside on the function of these cells and to explore the delivery of enzyme to them under rigorously controlled conditions. These cells are the site of stored pathological material in gaucher's disease and in a number of other metabolic disorders. Investigations with the patient's own cells provides the immense advantage that therapeutic strategies can be customized to the individual patient. Moreover, subtleties in genetic modifications and in pathogenetic aspects of these disorders can be examined with greater exactness with this technique than has been possible heretofore. We anticipate that progress in the treatment of these disorders will be greatly accelerated as a consequence of this strategically important development.

#### D. Delivery of Enzymes to the Central Nervous System

We have carefully examined the parameters and kinetics of the delivery of hexosaminidase A, the enzyme lacking in patients with Tay-Sachs disease, in rats and dogs following temporary alteration of the blood-brain barrier. We have confirmed our previous observation that enzymes can gain access to nerve cells in this fashion with  $^{125}\text{I}$ -labeled hexosaminidase. However, we feel that the quantity of enzyme delivered to the brain must be further increased before undertaking enzyme replacement in disorders that involve the central nervous system in humans. It is hoped that this impediment will soon be overcome since there is a growing number of patients on our register who require treatment and the enzymes for Tay-Sachs disease and for metachromatic leukodystrophy have now been successfully isolated from human tissue sources for replacement trials.

#### E. Development of Animal Models of Human Hereditary Disorders

Every investigator involved in the study of heritable metabolic disorders realizes the tremendous advantages that would become available if small animal analogues of human diseases could be produced. We have achieved some success in this regard using the following approaches to this goal. The first is the production of a pharmacological analogue of the mucopolysaccharide storage disorder known as Hunter's disease by the administration of the trypanocide suramin. Animals treated in this fashion show an inhibition of the same enzyme and the accumulation of identical substances that occur in the human disorder. The second development is the discovery of a spontaneous mutation in BALB/C mice that closely resembles human Niemann-Pick disease Type C. These animal models will be used to determine the point in time at which irreversible damage occurs in various tissues including that brain to indicate at what stage in development enzyme replacement must be carried out. It is expected that this information can be directly extrapolated to the situation in humans. These animal models will also be extraordinarily useful to explore the optimum mode of delivery, cell uptake, and catalytic efficiency of exogenous enzymes for the treatment of storage diseases.

## II. MEMBRANE RECEPTORS FOR ENVIRONMENTAL SIGNALS

It is well known that many environmental signals, including growth factors and hormones, exert their biological activity through their interaction with a receptor on the surface of cells which is coupled with the enzyme adenylate cyclase to increase intracellular cyclic AMP (cAMP). Significant progress has been made during the past year concerning the nature of the coupling mechanism between the receptor and this enzyme. The beta-adrenergic agonist isoproterenol and prostaglandin E<sub>1</sub> both increase cAMP. Repeated exposure of cells to these agents leads to a loss of cAMP production (desensitization). Previous experiments indicated that this phenomenon was due to loss of receptors in certain situations. However, our data with these agents indicate that there are important physiological changes in the components that link the receptor with adenylate cyclase. Isoproterenol causes an uncoupling between the receptor and a regulatory component that affects the activity of the cyclase. With prostaglandin, uncoupling occurs between the regulatory and catalytic components of adenylate cyclase. These studies should provide insight into the mechanism(s) involved in the unregulated growth of neoplastic cells.

## III. MULTIPLE SCLEROSIS

We have applied the highly sensitive radioimmunoassay procedure developed last year by us to measure the quantity of the major myelin-associated glycoprotein (MAG) in the brain of patients with multiple sclerosis. Using this technique, we have substantiated our previous immunohistochemical demonstration that MAG is selectively destroyed in areas of the brain at considerable distances from the multiple sclerosis plaque as well as within the plaque itself. The breakdown of MAG occurs long before histological alterations are detectable and before the loss of myelin basic protein can be demonstrated. We therefore believe that MAG is especially sensitive to pathologic alteration and information concerning its catabolism is important for understanding the pathogenesis of demyelinating disorders. Strong support for this assertion has been provided by examination of the catabolism of MAG and other myelin proteins in situ. We have discovered a neutral proteolytic enzyme in myelin itself that catalyzes the hydrolysis of MAG significantly more rapidly than any other myelin protein. Regulation of the activity of this protease appears to be extraordinarily important for maintaining the integrity of the myelin sheath and its participation in demyelinating diseases will be comprehensively examined.

## IX. ECTO-ENZYMES

Investigations concerning the physiologic importance of enzymes on the external surfaces of neural and other types of cells has accelerated significantly in the past year. Up to 70 percent of these membrane components are shed from the surface of cells in vitro in the form of microvesicles called exosomes. Covalent binding reagents were used to determine the kinetics of regeneration of these enzymes on cell membranes. The experiments indicated that recovery of catalytic activity occurs by the de novo synthesis of enzymes rather than by insertion of preformed enzymes into cell membranes. These findings have special significance with regard to the role of adenylates as modifiers of cellular excitability and the control of their physiological levels by the ectoenzymes that catalyze their breakdown. These enzymes and their adenylate substrates may play a role in the pathogenesis of traumatic shock. In addition, there is impressive evidence for their participation in the evolution of neurochemical transmission.

An important practical offshoot of these studies is the finding that manganese and magnesium activated ATPases on synaptic vesicles were severely inhibited by low levels of pesticides such as kepone, toxaphene, tricyclohexyltin, and triphenyltin hydroxides. Clearly the inhibition of these enzymes must be considered in the neurotoxicity of these widely used agents.

#### V. ENZYMATIC HYDROLYSIS OF AMYGDALIN (LAETRILE)

We have extended our investigation of the enzymes that catalyze the cleavage of the two molecules of glucose from amygdalin (Laetrile). These hexoses must be removed before cyanide, the putative tumoricidal agent, can be released from the molecule. We have examined two dozen fresh human tumor specimens and have not detected any activity of the required glucosidases. However, normal human small intestine and intestinal contents contain these enzymes. These findings indicate that amygdalin may not be a useful chemotherapeutic agent for cancer and they provide an explanation for the toxicity of orally ingested amygdalin.



## CONTRACT NARRATIVE

Developmental and Metabolic Neurology Branch  
Intramural Research Program, NINCDS  
October 1, 1980 through September 30, 1981

Contractor: NEW ENGLAND ENZYME CENTER, TUFTS UNIVERSITY (NO1-NS-5-2321)

Title: Preparation of Glucocerebrosidase from Human Placental Tissue

Contractor's Project Director: Henry E. Blair

Current Annual Level of Support: \$300,000

Objectives: To isolate human placental glucocerebrosidase in sufficient quantity and purity so that it can be used in enzyme replacement trials in patients with Gaucher's disease.

Major Findings: A procedure has been developed for the large-scale purification of human placental glucocerebrosidase that is of sufficient purity and catalytic activity that it can be safely administered to humans with Gaucher's disease. The intravenous infusion of this enzyme to four young patients with this disorder has brought about the following effects: (1) the progressive enlargement of the spleen and liver of these patients has been arrested. (2) The blood platelet count has been restored or maintained in the normal range. (3) The general health and vigor of the recipients has been dramatically improved.

Significance to Biomedical Research and to the Program of the Institute: One of the principal missions of the Institute is to develop effective therapies for the treatment of human diseases. If the results obtained in the initial trials of prospective enzyme replacement therapy discussed in the preceding paragraph can be extended and confirmed, we will have accomplished an unprecedented medical feat.

Proposed Course of the Contract: We plan to expand the number of recipients of enzyme replacement in Gaucher's disease to determine whether the initial salutary findings can be substantiated. We are also seeking to modify the enzyme so that it is more efficiently delivered to the specific cells that store the accumulating lipid. Finally, we shall investigate the possibility of altering the blood-brain barrier to try to deliver the enzyme to the central nervous system in patients with the neuronopathic form of this disease.

## CONTRACT NARRATIVE

Developmental and Metabolic Neurology Branch  
Intramural Research Program, NINCDS  
October 1, 1980 through September 30, 1981

Contractor: WEIZMANN INSTITUTE OF SCIENCE (N01-NS-0-2333)

Title: Production of Three Radiolabeled Glycolipid Substances

Contractor's Project Director: David Shapiro, Ph.D.

Current Annual Level of Support: \$56,000

Objectives: The enzymatic defects in heritable sphingolipid storage disorders in humans is ultimately best diagnosed through the use of radioactively labeled natural lipid substrates. The Weizmann Institute of Science provides the NIH with radioactive carbon-<sup>14</sup> labeled glucocerebroside, sphingomyelin, and ceramidetrihexoside for the diagnosis of patients and detection of carriers of Gaucher's disease, Niemann-Pick disease, and Fabry's disease respectively.

Major Findings: The principal investigator is a world-recognized expert in the chemical synthesis of sphingolipids. He has devised procedures for incorporating radioactive carbon-<sup>14</sup> into critical portions of sphingolipid molecules. Using these substrates, we incubate human tissue specimens to determine the activity of glucocerebrosidase sphingomyelinase and ceramidetrihexosidase enzymes. These determinations permit us to diagnose patients with the disorders listed above, to identify heterozygous carriers of these metabolic diseases, and to monitor pregnancies at risk for any of these conditions. These labeled lipids are also required to monitor the enzymes for therapeutic replacement trials.

Significance to Biomedical Research and to the Program of the Institute: The ability to diagnose patients, identify heterozygotes, and monitor pregnancies at risk for any of the known lipid storage diseases represents major contributions to the control of the incidence of the sphingolipidoses at the present time. Enzyme replacement appears to be of benefit to patients with Gaucher's disease and Fabry's disease.

Proposed Course of the Contract: The contractor will provide necessary radioactive sphingolipids for diagnostic tests and enzyme purification procedures. He will develop sphingolipid analogues for the production of animal models of human lipid storage diseases and he will prepare specific ligands for the purification of various sphingolipid hydrolases by affinity column chromatography.

## CONTRACT NARRATIVE

Developmental and Metabolic Neurology Branch  
Intramural Research Program, NINCDS  
October 1, 1980 through September 30, 1981

Contractor: NEW ENGLAND ENZYME CENTER, TUFTS UNIVERSITY (N01-NS-0-2339)

Title: Preparation of Ceramidetrihexosidase from Human Placental Tissue

Contractor's Project Director: Henry E. Blair

Current Annual Level of Support: \$80,933

Objectives: To isolate human placental ceramidetrihexosidase in sufficient quantity and purity so that it can be used in enzyme replacement trials in patients with Fabry's disease.

Major Findings: A procedure is being developed for the large-scale isolation of human placental ceramidetrihexosidase of sufficient purity and catalytic activity so that it can be safely administered to patients with Fabry's disease. Previous replacement trials with small quantities of this enzyme indicated that it catalyzed the clearance of accumulated lipid but that much larger quantities of the enzyme would be required in order to expect a beneficial clinical response. The necessary trials have been delayed by the presence of pyrogenic material(s) in larger batches of the enzyme; however, significant progress has recently been made by the contractor in eliminating this contaminant and it is anticipated that further clinical trials will soon be possible.

Significance to Biomedical Research and to the Program of the Institute:

A principal mission of the Institute is to develop effective procedures for the treatment of human diseases. If the early encouraging results with small quantities of enzyme can be extended and enlarged, it is expected that this form of treatment will be useful for Fabry patients.

Proposed Course of the Contract: We expect that larger quantities of pyrogen-free ceramidetrihexosidase will soon be made available by the contractor for clinical trials. If the results that are obtained are sufficiently promising, an adequate number of patients will be examined so that a reliable decision can be made concerning the effectiveness of enzyme replacement therapy in Fabry's disease.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 00706-22 DMN																														
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NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 50%;">John A. Barranger, M.D., Ph.D.</td> <td style="width: 20%;">Chief, Clinical Investigations and Therapeutics Section</td> <td style="width: 10%;">DMN</td> <td style="width: 10%;">NINCDS</td> </tr> <tr> <td>Other:</td> <td>George Constantopoulos, Ph.D.</td> <td>Research Biochemist</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>Daniel W. Stowens, M.D.</td> <td>Clinical Associate</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>Edward I. Ginns, M.D., Ph.D.</td> <td>Clinical Associate</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>Norman Barton, M.D., Ph.D.</td> <td>Clinical Associate</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>Roscoe O. Brady, M.D.</td> <td>Chief</td> <td>DMN</td> <td>NINCDS</td> </tr> </table>			PI:	John A. Barranger, M.D., Ph.D.	Chief, Clinical Investigations and Therapeutics Section	DMN	NINCDS	Other:	George Constantopoulos, Ph.D.	Research Biochemist	DMN	NINCDS		Daniel W. Stowens, M.D.	Clinical Associate	DMN	NINCDS		Edward I. Ginns, M.D., Ph.D.	Clinical Associate	DMN	NINCDS		Norman Barton, M.D., Ph.D.	Clinical Associate	DMN	NINCDS		Roscoe O. Brady, M.D.	Chief	DMN	NINCDS
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COOPERATING UNITS (if any)  None																																
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SUMMARY OF WORK (200 words or less - underline keywords) A better understanding of <u>metabolic disorders</u> which affect the nervous system is the goal of this project. In some phases, the studies are purely <u>diagnostic</u> and are applied to assist in diagnosing the <u>less common disorders of metabolism</u> . Other phases deal with biochemical observations in known disorders that suggest steps in the <u>pathogenesis</u> of the disease. In some poorly understood groups of <u>neurologic disease</u> , studies are conducted to draw biochemical correlations where none had previously been known or were poorly developed. <u>Therapeutic trials</u> are conducted in selected disorders.																																

Project Description:

Objectives: The majority of chronic neurological disorders are first recognized during childhood. Because of the chronicity and long duration of the handicaps these diseases constitute a formidable medical and social problem. Taken together, mental retardation (frequently familial), birth defects, cerebral degenerations, and inborn errors of metabolism affecting the nervous system amount to over four million people in the U.S.A. Our main objectives are: 1) to study the pathogenesis and etiology of these diverse disorders which are frequently of genetic origin, 2) to devise special diagnostic tests including identification of heterozygotes, 3) to institute therapeutic modifications of the respective disorders, 4) explore preventive measures and prenatal diagnosis.

Patient Material: Patients with the following disease categories were admitted for investigation; sphingolipidoses, mucopolysaccharidoses, ceroid lipofuscinosis, spinocerebellar degeneration, congenital pyruvic and lactic acidosis, glycogen storage disorders, and adrenoleukodystrophy. A number of patients with unknown diseases were admitted for study.

Methods Employed:

- 1) Neurologic, developmental and genetic assessments of the patients were made, including family studies when appropriate.
- 2) Determination of profiles of lipids, amino acids, proteins, mucopolysaccharides and carbohydrates in various tissues and, when appropriate, in urine and cerebrospinal fluid.
- 3) Assay of enzyme activities in the peripheral blood leukocytes and platelets of genetic diseases studied.
- 4) Establishment of skin fibroblast tissue cultures in patients with genetic disorders for study of enzyme activity and turnover studies using radioactive substances.
- 5) Employment of invasive techniques, if required, for definitive diagnosis. Brain and liver biopsies are performed and the tissues are used for biochemical, chemical, enzymatic and electron microscopic studies.
- 6) Therapeutic modification of the diseases is attempted whenever possible. For this purpose, pharmaceuticals, hormones, plasma or formed blood elements transfusion, dietary modifications and, where appropriate, enzyme replacement are used.
- 7) Post mortem samples of the organs and other tissues are stored frozen for future chemical and enzymatic studies; the fresh tissue is immediately fixed or processed for histochemical and electron microscopic studies.
- 8) Radio-assay of ganglioside  $\text{GM}_1$ .

Major Findings:

1. A portion of our efforts were directed toward an understanding of the pathogenesis of the mucopolysaccharidoses. Tissues were examined from a patient with the tentative diagnosis of lipidoses. Chemical findings



suggested a mucopolysaccharidosis. The tissues were found deficient in  $\alpha$ -N-acetylglucosaminidase and the correct diagnosis of MPS IIIB was made. Parallel findings have been made in isolated glial and neuronal fractions.

2. Concentrated efforts were made to introduce therapeutic modifications of selected inborn errors of metabolism. In an attempt to delineate the pathogenesis and clinical variability of the sphingolipidoses, and in order to proceed logically with therapeutic modalities, principally enzyme replacement, these disorders have been studied in depth. Gaucher's disease has been particularly closely scrutinized. Suggestions from the literature and observations of our patients have prompted us to investigate the significance of disturbances of liver function, lung function, immune response, cardiac function, and reticuloendothelial function. Results of some of these studies are cited in appropriate listed publications.
3. Patients presenting with myoclonus are being investigated. Four patients with the diagnosis of Lafora body disease have been identified. Clinicopathologic correlation has been made. The diagnostic value of the liver pathology has been confirmed. The nature of the biochemical defect is being investigated. Preliminary characterization of the storage material in liver and identification of a previously unknown urinary polysaccharide have been accomplished.
4. Patients with ataxia are being investigated for biochemical disorders. Two patients with ataxia have been demonstrated to have "ragged red fibers" in their muscle mitochondria. These patients have lactic and pyruvic acidemia and thus likely have some error of oxidative metabolism. No deficiency of the pyruvate dehydrogenase complex has been detected in these or other similar patients. The precise biochemical lesion is being pursued. Other causes of hereditary familial ataxia currently being actively tested are pyruvate dehydrogenase deficiency, hexosaminidase variants, and other variants of the sphingolipidoses. No patients with Friedreich's ataxia or olivopontocerebellar atrophy have been found to be deficient in any of these enzymes.
5. Modification of the blood-brain barrier results in the entry of macromolecules such as enzymes into brain interstitial fluid. We have further demonstrated that catalytically active enzymes are taken up and incorporated into lysosomes of neurons and to some extent glia. The procedure can be carried out safely and can be monitored noninvasively. The possibility of enzyme replacement in the central nervous system is being investigated. Furthermore, the receptors on neurons for macromolecules are being described. Studies designed to describe the processing of macromolecules by brain are being carried out.
6. Pilot studies in Fabry's disease indicate that the unmetabolizable lipid, ceramide trihexoside, can be removed by plasmapheresis. The kinetics of reappearance in the serum suggests that an exchangeable

pool of the lipid exists. Tissue concentrations of the lipid and clinical correlation will be made after multiple exchanges over a period of six months to one year.

7. As gangliosides are a part of all membranes, it was surmised that degenerative neurologic diseases would manifest an increased ganglioside content in the cerebrospinal fluid. This has been demonstrated using radioassay of ganglioside G<sub>M1</sub>.

#### Significance to Biomedical Research and the Program of the Institute:

Because the majority of infections affecting man are now under quite satisfactory control, the time has come for increased attention to accord a measure of control to such common disorders as hereditary diseases, congenital malformations, mental retardation, and degenerative conditions affecting the nervous system. Improved methodology makes it now feasible to advance our knowledge and institute some control in certain of these crippling chronic disorders. Prevention and therapy include prenatal diagnosis, enzyme infusion, dietary modifications and institution of certain eugenic measures. Since many of the disorders affect exclusively or predominantly the nervous system, the study of etiology and pathogenesis as well as institution of therapeutic trials are of importance in furthering the main mission of our Institute.

Proposed Course of the Project: The etiology of Friedreich's ataxia remains unknown. Recently it was reported that dichloroacetate activates the pyruvate dehydrogenase complex (PDHC) in tissue culture. This technique, by amplifying the differences between partially deficient and normal lines may help in establishing partial deficiencies and heterozygous states. We plan to take advantage of this method and to further scrutinize the abnormalities of pyruvate oxidation in the ataxias. In this regard, the examination of several enzyme systems in the clinically diverse category of spinocerebellar degenerations may provide some avenue of investigation of the hypothesis of abiotrophy.

The study of the regulation of PDHC in cultured cells is hampered by the frequent contamination with mycoplasmas. We find that several species of mycoplasmas have very high apparent PDHC activity. For better understanding the oxidative metabolism of pyruvate in fibroblasts we intend to investigate its oxidative metabolism in these simple organisms. During the next years increasing emphasis will be given to the pathogenesis of the hereditary diseases and to therapeutic modifications of respective disorders. We expect to evaluate the usefulness of enzymes in the treatment of various diseases. Further, the possibility of plasmapheresis as a therapeutic tool in storage disorders will be more thoroughly investigated. Dynamics of glycolipid metabolism will be appraised by estimation of the rates of synthesis and catabolism and the measurement of various pool sizes of the lipids.

Further study of the appearance and disappearance of ganglioside in degenerative diseases will be conducted.

Publications:

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2. Constantopoulos, G., Chang, C. S. C. and Barranger, J. A.: Normal pyruvate dehydrogenase complex activity in patients with Friedreich's ataxia. Ann. Neurol. 8: 636-639, 1980.
3. Brady, R. O. and Barranger, J. A.: Inborn lysosomal enzyme deficiencies. In: A. N. Davison and Thompson, R. H. S. (Eds.), The Molecular Basis of Neuropathology. London, Edward Arnold (Publishers) Ltd., 1980, in press.
4. Neuwelt, E. A., Barranger, J. A., Brady, R. O., Pagel, M., Furbish, F. S., Quirk, J. M., Mook, G. E. and Frenkel, E.: Delivery of hexosaminidase A to the cerebrum following osmotic modification of the blood-brain barrier. Proc. Natl. Acad. Sci. USA, 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 00815-21 DMN																		
PERIOD COVERED October 1, 1980 through September 30, 1981																				
TITLE OF PROJECT (80 characters or less)  Metabolism of Complex Lipids of Nervous Tissue																				
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																				
<table style="width: 100%; border: none;"> <tr> <td style="width: 60%;">PI: R. O. Brady, Chief</td> <td style="width: 10%;">DMN</td> <td style="width: 30%;">NINCDS</td> </tr> <tr> <td>OTHER: P. G. Pentchev, Biochemist</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td>A. E. Gal, Organic Chemist</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td>F. S. Furbish, Senior Staff Fellow</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td>J. A. Barranger, Section Chief</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td>A. D. Boothe, Veterinary Pathologist</td> <td>DMN</td> <td>NINCDS</td> </tr> </table>			PI: R. O. Brady, Chief	DMN	NINCDS	OTHER: P. G. Pentchev, Biochemist	DMN	NINCDS	A. E. Gal, Organic Chemist	DMN	NINCDS	F. S. Furbish, Senior Staff Fellow	DMN	NINCDS	J. A. Barranger, Section Chief	DMN	NINCDS	A. D. Boothe, Veterinary Pathologist	DMN	NINCDS
PI: R. O. Brady, Chief	DMN	NINCDS																		
OTHER: P. G. Pentchev, Biochemist	DMN	NINCDS																		
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A. D. Boothe, Veterinary Pathologist	DMN	NINCDS																		
COOPERATING UNITS (if any) Weizmann Institute of Science, Rehovot, Israel Tufts University Medical School, Boston, Massachusetts																				
LAB/BRANCH Developmental & Metabolic Neurology Branch																				
SECTION Enzymology and Genetics																				
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																				
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:																		
7.6	5.6	2.0																		
CHECK APPROPRIATE BOX(ES)																				
<input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER																				
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																				
SUMMARY OF WORK (200 words or less - underline keywords) Procedures have been developed for the purification of enzymes from human placenta that are lacking in patients with <u>Gaucher's disease</u> , <u>Fabry's disease</u> , <u>Tay-Sachs disease</u> and <u>Niemann-Pick disease</u> . The effects of <u>enzyme replacement therapy</u> in patients with these disorders is under investigation. Procedures have been developed for the <u>diagnosis</u> of patients with these disorders, the detection of heterozygous <u>carriers</u> of these genetic traits, and for the <u>monitoring</u> of pregnancies at risk for each of these diseases.																				

Project Description:

Objectives: (1) to elucidate the biosynthetic pathways for the formation of long chain fatty acids, cerebrosides and sphingomyelin gangliosides; (2) to study the control mechanisms which regulate these processes; (3) to study the metabolic fate of sphingolipids in normal and lipodystrophic disease states, and (4) to provide diagnostic and therapeutic procedures for the amelioration and control of the lipid storage diseases.

Methods:  $^{14}\text{C}$ -Glucocerebroside and  $^{14}\text{C}$ -galactocerebroside have been synthesized.  $^{14}\text{C}$ -labeled sphingomyelin, ceramidetrihexoside and gluco- and galactopycosine have been prepared. Ceramide tetra hexoside (globoside) specifically labeled with radioactive hydrogen- $^3\text{H}$  has been synthesized. The metabolism of these materials has been investigated *in vivo* and *in vitro*. Human placenta is a convenient and rich source of sphingolipid hydrolases. The isolation of enzymes that hydrolyze these sphingolipids for enzyme replacement trials is the principal continuing portion of this project.

Major Findings: (1) Enzyme replacement in Gaucher's disease and Fabry's disease holds promise as an effective therapeutic procedure for the amelioration of these disorders. A long lasting reduction of blood glucocerebroside, the accumulating lipid in Gaucher's disease, was observed in patients infused with purified human placental glucocerebrosidase. The purification of glucocerebrosidase on a large scale in a form that is suitable for administration to humans is now available. Enzyme replacement trials in Gaucher's disease are underway with this preparation. The clinical course of the disease was improved in four young boys who received the enzyme. Accordingly, they are being followed on a prospective long-term course of enzyme replacement. Other trials resulted in moderate to dramatic reductions in the quantity of accumulated glucocerebroside in the liver of four adult patients who received a course of corticosteroid prior to administration of the enzyme. Furthermore, pathologic bone changes in several Gaucher patients appear to have been improved by this therapeutic combination.

(2) We have developed a method for the purification of sphingomyelinase, the enzyme lacking in Niemann-Pick disease, also from human placental tissue. At the present time, it is very difficult to obtain sufficient quantities of this enzyme for replacement therapy trials. We have discovered a strain of Balb/c mice with an autosomal recessive neurological degenerative disorder characterized by the accumulation of several sphingolipids that closely resembles Niemann-Pick disease Type C in humans. We propose to utilize this animal model for studies of enzyme replacement therapy.

(3) We continue to serve as a center for the diagnosis of patients and detection of carriers for all of the lipid storage diseases and much of our effort is devoted to the monitoring of pregnancies at risk for heritable metabolic disorders. During the past year, we performed more than 300 diagnostic assays for physicians and genetic counselors from all over the world.



Significance: Enzyme replacement appears promising for the treatment of Gaucher's disease and Fabry's disease. It is expected that the deleterious clinical course in these patients will be ameliorated by this form of therapy. The ability to introduce enzymes into the central nervous system has profound implications for the treatment of genetic disorders that cause brain damage.

Proposed Course: We will continue to carry out and monitor the long-term effects of enzyme infusion in patients with Gaucher's disease, Fabry's disease and other disorders. Studies of enzyme replacement with purified sphingomyelinase will be carried out in animal models of Niemann-Pick disease. We shall design systems to deliver these exogenous enzymes to the cells in which the accumulating lipids are stored in order to improve the clinical efficiency of this form of therapy.

Publications:

1. Brady, R. O., Barranger, J. A., Gal, A. E., Pentchev, P. G., Furbish, F. S., and Kusiak, J. W. Treatment of lipidoses by enzyme infusion. In Lowden, J. A. and Callahan, J. W. (Eds.): Lysosomes and Lysosomal Storage Diseases, New York, Raven Press, 1981, pp. 373-379.
2. Morrone, S., Pentchev, P. G., Baynes, J., and Thorpe, S.: Studies in vivo of the tissue uptake, cellular distribution and catabolic turnover of exogenous glucocerebrosidase in rat. Biochem. J. 194: 733-742, 1981.
3. Pentchev, P. G., Gal, A. E., Booth, A. D., Omodeo-Sale, F., Fouks, J. Neumeyer, B. A., Quirk, J. M., Dawson, G., and Brady, R. O.: A lysosomal storage disorder in mice characterized by a dual deficiency of sphingomyelinase and glucocerebrosidase. Biochim. Biophys. Acta 619: 669-679, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 01309-16-DMN												
PERIOD COVERED October 1, 1980 through September 30, 1981														
TITLE OF PROJECT (80 characters or less) Biosynthesis and Function of Glycosphingolipids and Other Glycoconjugates														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 70%;">PI: P. H. Fishman, Chief, Membrane Biochemistry Section</td> <td>DMN, NINCDS</td> </tr> <tr> <td>OTHER: R. V. Rebois, NIH Fellow</td> <td>DMN, NINCDS</td> </tr> <tr> <td>H. Miller-Podraza, Visiting Fellow</td> <td>DMN, NINCDS</td> </tr> <tr> <td>S. K. Beckner, Ph.D., Staff Fellow</td> <td>DMN, NINCDS</td> </tr> <tr> <td>D. R. Critchley, Visiting Scientist, Dept. of Biochemistry, University of Leicester, England</td> <td></td> </tr> <tr> <td>R. O. Brady, Branch Chief</td> <td>DMN, NINCDS</td> </tr> </table>			PI: P. H. Fishman, Chief, Membrane Biochemistry Section	DMN, NINCDS	OTHER: R. V. Rebois, NIH Fellow	DMN, NINCDS	H. Miller-Podraza, Visiting Fellow	DMN, NINCDS	S. K. Beckner, Ph.D., Staff Fellow	DMN, NINCDS	D. R. Critchley, Visiting Scientist, Dept. of Biochemistry, University of Leicester, England		R. O. Brady, Branch Chief	DMN, NINCDS
PI: P. H. Fishman, Chief, Membrane Biochemistry Section	DMN, NINCDS													
OTHER: R. V. Rebois, NIH Fellow	DMN, NINCDS													
H. Miller-Podraza, Visiting Fellow	DMN, NINCDS													
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D. R. Critchley, Visiting Scientist, Dept. of Biochemistry, University of Leicester, England														
R. O. Brady, Branch Chief	DMN, NINCDS													
COOPERATING UNITS (if any) Laboratory of Cellular Metabolism, NHLBI Laboratory of Molecular Biology, NCI Laboratory of Biochemical Pharmacology, NIADDK														
LAB/BRANCH Developmental & Metabolic Neurology Branch														
SECTION Membrane Biochemistry														
INSTITUTE AND LOCATION NINCDS, Bethesda, Maryland 20205														
TOTAL MANYEARS: <div style="text-align: center;">3.7</div>	PROFESSIONAL: <div style="text-align: center;">3.1</div>	OTHER: <div style="text-align: center;">0.6</div>												
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS   <input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS         </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div>														
SUMMARY OF WORK (200 words or less - underline keywords) <p> <u>Gangliosides</u> appear to be important recognition molecules on the <u>cell surface</u>. 1. The membrane receptor for cholera toxin is believed to be the ganglioside <math>G_{M1}</math>. We have directly demonstrated that the receptor for cholera toxin on rat intestinal brush borders is <math>G_{M1}</math> and glycoproteins. 2. Previous studies suggested that gangliosides may be involved in the binding and action of <u>thyrotropin</u> to <u>thyroid</u> cells. Using a clonal line of normal rat thyroid cells, we have been able to demonstrate that gangliosides do not play a role in thyrotropin binding or action. 3. Gangliosides inhibit the attachment of cells to <u>collagen</u> which is mediated by fibronectin, a cell surface glycoprotein. Cells that are deficient in gangliosides synthesize but do not retain fibronectin. When the cells are treated with gangliosides, the cells retain fibronectin and organize it into fibrillar arrays. 4. Cultured cells incorporate radioactive precursors into their gangliosides within 5 min, but the labeled gangliosides do not appear on the cell surface until after 30 min. This lag appears to represent the time required for the newly synthesized gangliosides to be translocated from the <u>Golgi apparatus</u> to the <u>plasma membrane</u>.         </p>														

16 - DMN/IRP

## Project Description

**Objectives:** To investigate the function of membrane glycosphingolipids in the regulation of cell proliferation, cell morphology, hormone action and toxin sensitivity; to explore the regulation of glycosphingolipid biosynthesis during development and differentiation and relate these findings to metabolic disorders; to determine the underlying mechanism of altered glycosphingolipid biosynthesis in neoplasia; these studies are being extended to other membrane glycoconjugates.

**Methods:** The glycosphingolipid composition of cultured cells and tissues is determined by extraction and purification of this class of lipids followed by separation of individual glycolipids on thin-layer chromatograms. Metabolism in cultured cells is determined by adding radiolabelled precursors to the culture medium and isolating the labelled glycosphingolipids. Biosynthesis *in vitro* is analyzed by assaying the activities of the glycosyltransferases involved in glycosphingolipid synthesis. Surface glycoconjugates are labelled by selective oxidation with galactose oxidase or periodate and subsequent reduction with sodium borotritide. Gangliosides radiolabeled in specific portions of the molecule are prepared by specific enzymatic and chemical reactions.

Binding of toxins and hormones to cells and cell membranes is determined with [<sup>125</sup>I]-labeled proteins and either filtration or centrifugation techniques. Levels of cyclic AMP and adenylate cyclase activity are measured with a modified cyclic AMP protein binding assay.

### Major Findings:

#### A. Interaction of Fibronectin with Gangliosides

Fibronectin is a major cell surface glycoprotein which mediates cell attachment and is diminished in many malignant cells. Fibronectin binds to a specific region on collagen and thus mediates the attachment of fibroblasts to the collagen substratum. Fibronectin appears to be loosely associated with the cell surface and the nature of the surface binding sites for fibronectin are not yet known. We have found that gangliosides block the fibronectin-mediated attachment of cells to collagen-coated culture dishes. These observations were explored further using NCTC 2071 cells, a line of transformed mouse fibroblasts that are unable to synthesize gangliosides and that can grow in serum-free medium. Using immunofluorescence, the cells were found to be able to make fibronectin but not retain it on their surfaces. When the cells were cultured in medium containing gangliosides, the cells now retained fibronectin and organized it into fibrillar arrays. Complex gangliosides such as G<sub>Q1a</sub> were more effective than simple ones such as G<sub>M3</sub>. Since previous studies had demonstrated a loss of complex cellular gangliosides from many malignant transformed cells, there may be a coordinate loss of cellular adhesion components that potentiates abnormal behaviour both in culture and *in vivo*.

#### B. Receptors for Cholera Toxin in Rat Intestinal Brush Borders

Previous studies have established that G<sub>M1</sub> can function as a cell surface receptor for the *Vibrio cholerae* toxin, which mediates its effects by irreversibly activating adenylate cyclase. Little is known about the toxin receptors in intestinal mucosa, the natural target of the toxin. Work by others implicated intestinal glycoproteins as receptors for cholera toxin.

We decided to re-examine this issue. We were able to show that 99% of the toxin receptors were extracted from rat intestinal brush borders with chloroform/methanol solutions. On thin-layer silica gel, the receptors migrated as  $G_{M1}$ . Prior incubation of the membranes with cholera toxin protected the  $G_{M1}$  but not the glycoproteins from galactose oxidase. Toxin-receptor complexes extracted from the membranes with detergents sedimented on sucrose density gradients as toxin- $G_{M1}$  complexes and not as putative toxin-glycoprotein complexes. Membranes were dissolved in SDS and separated by SDS polyacrylamide gel electrophoreses. When the gels were overlaid with  $^{125}I$ -toxin, the toxin only bound to the region of the gels containing lipids and not to any of the glycoproteins. We conclude that  $G_{M1}$  is the predominant if not only receptor for cholera toxin in the intestine.

### C. Role of Gangliosides in Thyrotropin Binding and Action

In a number of previous studies, evidence was presented that indirectly implicated gangliosides as receptors for the hormone, thyrotropin (TSH), and as possible mediators of TSH action on thyroid cells. We decided to develop a more direct approach to address this hypothesis. Studies were initiated with a clonal line of normal rat thyroid cells. We were able to show that these cells bound small amounts of TSH with high affinity and specificity under physiological conditions. The cells also contained a large number of low affinity sites for TSH. TSH stimulated the production of cyclic AMP by the cells and the  $K_a$  was the same as the  $K_d$  of the high affinity binding site. Thus, the high affinity TSH receptors appeared to functionally coupled to adenylate cyclase. The cells also bound and responded to cholera toxin. The major ganglioside of the cells was  $G_{M3}$  with trace amounts of  $G_{M1}$  and  $G_{D1a}$ . When treated with neuraminidase, most of the  $G_{D1a}$  was converted to  $G_{M1}$ ; the cells bound much more cholera toxin but only slightly more TSH. When the neuraminidase-treated cells were incubated with the B component of cholera toxin, binding of toxin, but not TSH, was blocked. Prior treatment of thyroid cells with gangliosides caused a 10-fold increase in toxin binding but no change in TSH binding. There was no effect on TSH responsiveness when cells were either pretreated with gangliosides or gangliosides were added to the incubation. Finally, prolonged exposure of the thyroid cells to TSH resulted in loss of TSH receptors (down-regulation). There was no change, however, in either surface gangliosides or toxin receptors. These results indicate that gangliosides are not involved in TSH binding or action. Previous studies implicating gangliosides were done under non-physiological conditions where TSH binds predominantly to low affinity sites on the thyroid membrane.

### D. Biogenesis of Gangliosides in Cultured Cells

Neuroblastoma cells contain large amounts of gangliosides  $G_{M3}$ ,  $G_{M2}$ ,  $G_{M1}$ , and  $G_{D1a}$ . The ratio of cholera toxin binding to  $G_{M1}$  content in two different clones, N18 and NB, was 6-7. The N18 cells contained 20-fold more  $G_{M1}$  than NB cells. As each toxin molecule theoretically binds to 5  $G_{M1}$  molecules, these results indicate that most of the  $G_{M1}$  is on the cell surface. When the cells were treated with neuraminidase, there was a parallel increase in both  $G_{M1}$  content and toxin binding. The levels of  $G_{D1a}$  and  $G_{M3}$ , which are susceptible to neuraminidase, decrease by 75-80% whereas  $G_{M2}$  remained unchanged. Cells incubated in medium containing  $[^3H]$  galactose or palmitate incorporated label into their gangliosides within 5 min. We developed a method to distinguish between cell surface and internal gangliosides. Cells were



labeled as above for different times and incubated with sodium periodate at 4°C in order to oxidize sialic acid residues on surface gangliosides. Gangliosides were isolated and treated with dinitrobenzylhydrazine, which reacts only with the oxidized gangliosides. The dinitrophenol derivatives were separated from native gangliosides by thin-layer chromatography and analyzed for radioactivity. Using this procedure, we found that there was a 30 min lag before labeled gangliosides appeared on the cell surface. Although the bulk of the gangliosides are on the cell surface, they are synthesized inside the cell (presumably in the Golgi apparatus) and then translocated to the cell surface by yet undefined processes.

Significance: These studies are providing information on the function of membrane glycosphingolipids. Ganglioside G<sub>M1</sub> has been shown to be the receptor for cholera toxin in intestinal mucosa. Other gangliosides may provide binding sites for fibronectin, a cell adhesion protein.

Proposed Course: The project will be continued with emphasis placed on the role of gangliosides in cell attachment. Further work will be done on the biosynthesis of gangliosides and their translocation to the cell surface.

#### Publications:

1. Beckner, S. K., Brady, R. O. and Fishman, P. H.: A re-evaluation of the role of gangliosides in the binding and action of thyrotropin. Proc. Natl. Acad. Sci. USA 78: in press, 1981.
2. Fishman, P. H.: Membrane changes in virally transformed cells. In Blough, H. A. and Tiffany, J. M. (Eds.): Cell Membranes and Viral Envelopes. London, Academic Press, 1980, 331-374.
3. Fishman, P. H., Pacuszka, T., Hom, B. and Moss, J.: Modification of ganglioside G<sub>M1</sub>: effect of lipid moiety on cholera toxin action. J. Biol. Chem. 255: 7657-7664, 1980
4. Rebois, R. V., Omedeo-Sale, F., Brady, R. O. and Fishman, P. H.: Covalent crosslinking of human chorionic gonadotropin to its receptor in rat testes. Proc. Natl. Acad. Sci. USA 78: 2086-2089, 1981.
5. Critchley, D. R., Magnani, J. L. and Fishman, P. H.: Interaction of cholera toxin with rat intestinal brush border membranes: relative roles of gangliosides and galactoproteins as toxin receptors. J. Biol. Chem. 256: in press, 1981



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01457-15 DMN
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less) The Chemical Synthesis of Radioactive Sphingolipids		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <div style="display: flex; justify-content: space-between;"> <div>           PI: A. E. Gal, Chief, Neurochemical Methodology Section            OTHER: F. J. Fash, Bio. Lab. Technician         </div> <div style="text-align: right;">           DMN, NINCDS            DMN, NINCDS         </div> </div>		
COOPERATING UNITS (if any)  None		
LAB/BRANCH Developmental and Metabolic Neurology Branch		
SECTION Neurochemical Methodology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Md. 20205		
TOTAL MANYEARS: 0.6	PROFESSIONAL: 0.3	OTHER: 0.3
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS   <input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS         </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) Sphingolipids containing radioactive isotopes were synthesized and used for metabolic studies and as diagnostic tools in sphingolipidoses. <sup>14</sup> C and <sup>3</sup> H labels were introduced by synthetic and semi-synthetic techniques, gas exposure, and a new approach: functional group exchange.		

Project Description:

Objectives: To prepare sphingolipids labeled with radioactive isotopes. The compounds are used for metabolic studies and as diagnostic tools in investigations related to hereditary lipid storage diseases.

Methods and Major Findings: A multitude of approaches were used in labelling glycolipids such as chemical synthesis, partial synthesis, minor synthetic modifications, functional group exchange and tritium gas exposure. These methods could be classified into two categories: specific and non-specific labelling. The ideal approach is the specific labelling which consists of the tagging of a complex molecule at a predetermined atom. Total synthesis is the best way to accomplish this but up to now only few sphingolipids have been synthesized. We synthesized sphingosine, psychosine and galactocerebroside specifically labelled by total synthesis. However, our main effort is directed toward methods which would allow specific labelling of atoms yet would not necessitate tedious syntheses. A promising technique which we developed is called the functional group exchange. A chemical group such as an acetyl or carboxyl is split from a molecule and is replaced with a similar but radioactive one. With this approach we could prepare aminosugars, even gangliosides. Using the approach minor synthetic modification we prepared asialo ganglioside, Tay-Sachs ganglioside and ceramidetrihexoside. In this approach oxidation and reduction of an alcohol group in the molecule with a radioactive reducing agent would reestablish the original lipid in radioactive form. The lipids used as starting material for this approach were isolated from human tissues. Tritium gas exposure, a non-specific approach, was repeatedly used for labelling ceramide dihexoside, dihexoside and globoside. By this method all the non-labile hydrogen atoms in a molecule become radioactive. This procedure is relatively simple but the purification of the resulting compounds is complex. Also this type of compound requires more elaborate extractions for enzyme assays.

L-glucosylceramide was synthesized. This compound is a stereoisomeric analogue of D-glucosylceramide that occurs in nature and accumulates in pathological quantity in the organs and tissues of patients with Gaucher's disease. The properties of L-glucosylceramide that have been examined so far have been found to be indistinguishable from the naturally occurring glycolipid. However, L-glucosylceramide is completely refractory to enzymatic hydrolysis by purified placental glucocerebrosidase and enzyme(s) present in whole tissue extracts.

Significance: The compounds are indispensable for the detection, identification and isolation of enzymes connected to lipid storage diseases. Also studies related to qualitative and quantitative determination of enzymes in animal or human tissues necessitate these labelled substrates. Prenatal diagnoses are of rising importance. These labelled compounds play a key role in these diagnostic procedures. As a therapeutic approach, this branch initiated replacement therapy by the administration of the missing enzyme in hereditary diseases. The monitoring of the enzyme levels during and after this therapeutic procedure was done by the use of these radioactive substrates. It would be also of great interest to develop new methods which would allow relatively easy and inexpensive preparation of these compounds for the use of clinicians and for researchers who are not connected to a large research center. It is anticipated that L-glucosylceramide will be a uniquely useful substance for exploring pathogenetic processes in animal analogues of Gaucher's disease.

Proposed Course: Work on this project continues in three major directions: 1. Glycolipids will be labeled by using the above mentioned techniques with  $^{14}\text{C}$  and Tritium. 2. The approach using "minor synthetic modification" will be extended and used on lipids which were not prepared at all or not prepared by this technique. Also the replacement of the enzymatic oxidation will be explored. 3. Work will continue on the development of the technique: labeling by functional group exchange. 4. Work will continue on the synthesis of glycolipids which contain radioactive L carbohydrates instead of the naturally occurring D enantiomer such as L-galactocerebroside. It is anticipated that these lipids will be uniquely useful for exploring pathogenic processes in glycolipidoses.

Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do <b>NOT</b> use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 01480-14 DMN
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less) Metabolism of Neurohumoral Transmitter Substances in Marine Animals		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: E. G. Trams, Chief, Physiology and Metabolism Section DMN, NINCDS OTHER: N. Salem, Staff Fellow DMN, NINCDS C. Lauter, Chemist DMN, NINCDS J. Doherty, Toxicology Branch, EPA A. A. Benson, Biology Prof., Scripps Institute of Oceanography		
COOPERATING UNITS (if any) Mote Marine Lab., Sarasota, Florida Hazard Evaluation Division, Environmental Protection Agency, Washington, D. C. Scripps Institute of Oceanography, LaJolla, CA.		
LAB/BRANCH Developmental and Metabolic Neurology Branch, DMN		
SECTION Physiology and Metabolism		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD. 20205		
TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.3	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  The purpose of this project is to explore the great variety and abundance of the <u>marine environment</u> for <u>molecular models of neurobiology</u> . In particular it was designed to investigate species or phenomena which display an amplification or simplification of human physiologic or pathologic metabolism. Further studies were conducted on the neurotoxic effects of several pesticides in tissue preparations derived from lobster axons and from mammalian brains. Comparative studies in a variety of species dealt with the role of divalent cations in the activation of membrane ATPases. It was found that $Mn^{2+}$ plays a dominate role in ATPase activation in particular in the lower (invertebrate) species. In mammalian brains $Mn^{2+}$ appears to be the most active cation for the activation of ATPases in synaptic vesicles.		

Project Description:

Objectives: To explore by experimentation in comparative biochemistry certain aspects of neurobiology that can be studied more easily and profitably in other than common laboratory species. Typical experimental models which have exploited the great variety or biochemical specialization of marine life forms are squid giant axon, torpedo electroplax and tetrodotoxin. Specifically, we use comparative studies to study the role of ectoenzymes and adenylates in the modulation of cellular excitability.

Methods: A fractionation process allowed us to prepare plasma membranes from walking leg axons of the lobster (Homarus americanus). The membrane fraction was characterized by assaying for membrane marker enzymes. Enzyme assay were generally conducted with isotopic substrates. For comparative studies of ecto-enzyme activity, a variety of other species were also used, e.g. annelids (glycera), sea urchins (lytechinus), amoebae, yeasts and some lower vertebrates.

Major Findings:

Ecto-ATPase activity of intact cells was determined in a variety of cell types and in various species. Species variation for enzyme activity was substantial. The most active ecto-ATPases were found in avian erythrocytes where specific activity is about one thousand-fold greater than in man. In invertebrates (e.g. amoebae, yeasts) the divalent cation activated ATPases were most prominently stimulated by the  $Mn^{2+}$ , while at similar concentrations,  $Mg^{2+}$  and  $Ca^{2+}$  were less effective. An ATPase which was optimally stimulated by  $Mn^{2+}$  was also found in synaptic vesicles of mammalian brains. At higher  $Mn^{2+}$  concentrations, the enzyme was inhibited and it is possible that chronic  $Mn^{2+}$ -toxicity is related to variations in the intercellular or cytoplasmic concentrations of this divalent cation.

In a collaborative study we also investigated the effects of several pesticides on ATPase activity. We addressed this question, because of the known chronic (and sometimes acute) toxicity of pesticides which generally belong to the chlorinated hydrocarbon class. Divalent cation ATPases in axonic membrane preparations derived from the lobster Homarus americanus contained separate  $Mn^{2+}$  and  $Ca^{2+}$  stimulated ATPases.  $Mn^{2+}$  ATPase was found to be most active at 20°C whereas  $Ca^{2+}$  ATPase was most active at 37°C. Divalent cation ATPases were inhibited by the pesticides DDT, kepone, plictran and allethrin with  $IC_{50}$  values ranging from  $10^{-6}$  to  $10^{-4}M$ . Oligomycin ( $IC_{50} < 10^{-7}M$ ) was found to be a potent inhibitor. Mn and Mg ATPases in synaptic vesicles were inhibited to the same degree but showed a wide range of  $I_{50}$ 's for several pesticides. Examples are: Kepone and toxaphene (both  $I_{50}$ 's  $= 5 \times 10^{-6}M$ ), DDT ( $5 \times 10^{-5}M$ ), tri-cyclohexylin and triphenyltin hydroxides (both  $3 \times 10^{-7}M$ ). The organophosphate parathion ( $5 \times 10^{-5}M$ ) and oligomycin ( $10^{-7}M$ ) were not inhibitory. These data indicate that inhibition of Mn and Mg sensitive synaptic vesicle associated ATPases must be considered in addition to inhibition of mitochondrial and of Ca-dependent ATPases when evaluating neurotoxic mechanisms for pesticides which inhibit ATPase activity.



Proposed Course: We propose to extend our comparative studies on ecto-enzymes to several other species. It is possible that the very high ecto-ATPase activity in avian nucleated erythrocytes is related to metabolic regulation of the vascular bed; we propose to sample several other avian species to test this hypothesis. Further comparative studies will be made with unicellular organisms and with fishes, amphibians and reptiles. We propose to continue our investigations with crustacean axon membranes. Experiments which relate to the role of polychlorinated hydrocarbon pesticides to neurotoxicity and the role of chronic  $Mn^{2+}$  exposure will be continued.

Publications:

1. Doherty, J.D., Salem, N., Lauter, C. J. and Trams, E.G.:  $Mn^{2+}$  and  $Ca^{2+}$  ATPases in lobster axon plasma membranes and their inhibition by pesticides. Comp. Biochem. Biophys. (in press).
2. Trams, E. G.: On the evolution of neurochemical transmission. Differentiation (in press).
3. Trams, E. G., Lauter, C. J. and Salem, N.: Interspecies variation of divalent cation-activated ecto-ATPases. Comp. Biochem. Biophys. 69B: 195-199, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 01487-14 DMN
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Studies on the Composition and Metabolism of Cellular Membranes		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: E. G. Trams, Chief, Physiology and Metabolism Section, DMN NINCDS OTHER: N. Salem, Staff Fellow DMN NINCDS C. Lauter, Chemist DMN, NINCDS E. MacDonald, Visiting Fellow DMN, NINCDS M. Reinila, Guest Worker DMN, NINCDS S. Patton, Professor, University of California, San Diego, CA.		
COOPERATING UNITS (if any) Unit on Neurochemistry, CPB, NIMH Dept. of Neurosciences, Univ. of California, San Diego, CA.		
LAB/BRANCH Developmental & Metabolic Neurology Branch		
SECTION Physiology and Metabolism		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD. 20205		
TOTAL MANYEARS:  <div style="text-align: center;">3.9</div>	PROFESSIONAL:  <div style="text-align: center;">3.9</div>	OTHER:  <div style="text-align: center;">0</div>
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINDS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The objective of this project is to elucidate the relationship between molecular composition and topographic arrangements of membrane building blocks with reference to plasma membrane function. Bioelectrogenesis, transport and many metabolic phenomena are based on the proper associations of membrane proteins and lipids. Membrane ecto-enzymes are glycoproteins and require a lipophilic environment for optimal activity. Ecto-phosphoesterhydrolases appear to be a part of a regulatory system which modulates membrane permeability and excitability. Using covalently reacting chemical probes we have been able to achieve selective modifications of membrane ecto-enzymes. <u>Ecto-ATPases</u> and <u>ecto-5'-nucleotidases</u> in particular have been the subject of these investigations. These enzymes are embedded in the lipophilic environment of the plasma membrane, but project their catalytic sites into the extracellular environment. Selective modifications of these enzymes are synthesized <u>de novo</u> and inserted into the membrane. Specific domains of the plasma membrane are exfoliated in the form of microvesicles (exosomes) which contain the ecto-enzymes as markers.		

Project Description:

Objectives: To elucidate the molecular composition and topographic arrangement of membrane building blocks and their role in defining the physiologic functions of the plasma membrane. Furthermore, to inquire if cell pathology in certain diseases of the CNS is associated with derangements of the function of such membrane components.

Methods: A variety of cultured cell lines derived from the CNS are employed. Established clones of neuroblastoma, glioma and several primary cultures are maintained in monolayer cultures. Enzyme activities on the monolayer cultures are primarily assayed by the use of radiolabeled substrates. In addition, High Performance Liquid Chromatography is used to follow transition of metabolic products through various compartments. Covalently reacting chemical probes are used to label cell surface constituents *in situ* and differential labeling is achieved by substrate protection of the respective enzymes. Lipids and proteins so labeled are analyzed after separation by classical techniques. Exosomes are harvested by ultracentrifugation or by filtration. Interaction of exosomes with cells is measured by labeling exosomes with radioisotopes. Human erythrocyte ghosts are prepared from whole blood samples of normal volunteers and of manic-depressive patients. Variations in  $Mg^{2+}$ -ATPase,  $Na^+$ ,  $K^+$ -ATPase and  $Ca^{2+}$ -ATPase are measured in an attempt to define the absolute levels and their variance in control subjects and in patients.

Major Findings:

1. We have treated intact central nervous system cells with chemical probes which react covalently with proteins and aminophospholipids. Selective alterations of the enzymatic activities of ecto-ATPases, ecto-5'-nucleotidases and cholinesterases were obtained under appropriate reaction conditions. The cross-linking reagent, 1,5-difluoro-2,4-dinitrobenzene, was a potent inactivator of ecto-ATPase of C6 glioblastoma, IMR-32 neuroblastoma and of a primary rat astroblast cell line (RB). Ecto-5'-nucleotidase and acetylcholinesterase were less sensitive to difluorodinitrobenzene. 1-Fluoro-2,4-dinitrobenzene at concentrations which inactivated ecto-ATPase had little effect on ecto-5'-nucleotidase. Conversely, 2,4,6-trinitrobenzenesulfonic acid was a potent inactivator of ecto-5'-nucleotidase but had no effect on ecto-ATPase. The difluorodinitrobenzene inactivation of ecto-ATPase and of ecto-5'-nucleotidase as well as the fluoro-dinitrobenzene inactivation of ecto-ATPase could be prevented by the presence of the appropriate substrates in the reaction medium. In the presence of protecting nucleotide substrates, a decrease in reactivity with proteins and lipids was observed when the isotopic probe fluorodinitro-[ $^3H$ ]-benzene was used.

2. Cultures from various normal neoplastic cell lines exfoliated vesicles with 5'-nucleotidase activity which reflected the ecto-enzyme activity of the parent monolayer culture. The ratio of 5'-nucleotidase to ATPase activity in the microvesicles indicated that cellular ecto-ATPase was conserved in the exfoliative process. Phospholipids of the microvesicles contained significantly increased amounts of sphingomyelin and total polyunsaturated fatty acids. It was concluded that the shedded vesicles constituted a select portion of the plasma membrane. The vesicles had an average diameter of 500 to 1000 nm and often contained a second population of vesicles about 40 nm in diameter. As much as 70% of the plasma membrane

ecto-5'-nucleotidase activity of a culture was released into the medium over a 24-h period. Phosphoesterhydrolases from C-6 glioma or N-18 neuroblastoma microvesicles dephosphorylated cell surface constituents when in contact with monolayer cultures. Exfoliated membrane vesicles may serve a physiologic function; it is proposed that they be referred to as exosomes.

3. C6 glioblastoma cells express about half of the 5-nucleotidase activity as an ecto-enzyme. The membrane impermeant trinitrobenzenesulfonic acid (TNBS) irreversibly reacts with and inactivates ecto-5'-nucleotidase and was used to study 5'-nucleotidase metabolism and its regulation. We found that 70-80% of ecto-5'-nucleotidase activity was lost after this treatment but that the cells were viable as measured by trypan blue staining, containment of intracellular LDH, incorporation of  $^3\text{H}$ -thymidine into DNA, and cell growth. After 24 hrs of incubation with growth medium C6 cell ecto-5'-nucleotidase activity had nearly recovered ( $88 \pm 4\%$  of control). Fetal calf serum was not necessary for recovery as cells deprived of serum for three days also recovered activity. The rate of increase in ecto-5'-nucleotidase activity of TNBS treated cells exceeded that of control cells but there was no general increase in membrane biosynthesis as evidenced by normal specific binding of  $^{125}\text{I}$ -cholera toxin to  $\text{GM}_1$ . There was no recovery of surface activity in the presence of the protein synthesis inhibitor cycloheximide (0.2  $\mu\text{g}/\text{ml}$ ) but recovery began when cycloheximide was removed. TNBS treatment causes a concomitant loss in exosomal 5'-nucleotidase during the active recovery period but exfoliated enzyme activity approaches normal levels as the membrane enzyme recovers. Exosome production is normal during the recovery period as measured by  $\text{GM}_1$  and protein content. Our data indicate that ecto-5'-nucleotidase replacement occurs via de novo synthesis rather than by insertion of pre-formed enzyme. Decrease in exfoliated 5'-nucleotidase activity indicates either release of inactivated plasma membrane enzyme or a selective conservation of the enzyme in the exfoliative process.

4. Several purines were shown to be competitive inhibitors of [ $^3\text{H}$ ] diazepam binding. Inosine has also been shown to have benzodiazepine-like neurophysiologic, pharmacologic and behavioral effects, and to partially inhibit caffeine-induced seizures in mice. Using presumptive therapeutic doses, inosine levels were determined in mouse brain at various times following injection. Inosine and hypoxanthine concentrations in brain increased several fold following inosine administration indicating that it readily permeated the blood-brain barrier. The levels of inosine and hypoxanthine attained in brain were sufficient to inhibit by more than 50% the GABA stimulated [ $^3\text{H}$ ] diazepam binding. The anticonvulsant properties of inosine may be related to its interaction with the benzodiazepine receptor.

5. Preliminary studies on erythrocyte ATPases indicated that these enzymes not only occurred at various phenotypic levels in the RBC of normal human volunteers, but that they also may be subject to diurnal or circadian changes in activity levels. Most of our effort has been directed towards establishing reproducible laboratory procedures for the assay of human erythrocyte ATPases, prior to measurements in manic-depressive patients.



### Proposed Course

We will continue our investigations on the biological function of ecto-enzymes. It now seems probable that these biocatalysts have a multifunctional role and experiments will be designed to interrogate for probable functions in the appropriate model systems. Some of our studies shall address themselves to the question whether or not there is a significant correlation of ecto-ATPase or ecto-5'-nucleotidase with neoplasia, malignancy or differentiation. Another inquiry will be directed at the problem of the relationships between ectopic adenylates and ecto-enzymes in cellular excitability. Selective chemical modification of plasma membrane enzymes will be used to study membranogenesis. Further investigations on the biological significance of exosome production are under way; in particular, their possible function as transport vehicles in vivo will be studied.

### Significance

Our investigations have provided strong support for a role of adenylates as cellular excitability modifiers and for ecto-ATPase and ecto-5'-nucleotidase as regulators of this activity. The discovery of exfoliation of membrane vesicles (exosomes) suggests a new mode of intercellular communication or transport function.

### Publications:

1. Salem, N., Lauter, C.J. and Trams, E. G.: Selective Chemical Modification of plasma membrane ecto-enzymes. Biochim. Biophys. Acta 641: 366-376 1981.
2. Salem, N., Serpentino, P., Puskin, J. S. and Abood, L. G.: Preparation and spectroscopic characterization of molecular species of brain phosphatidylserines. Chem. & Physics of Lipids 27: 289-304 1980.
3. Marangos, P. J., Trams, E. G., Clark-Rosenberg, R. L., Paul, S.M. and Skolnick, P.: Anticonvulsant doses of inosine result in brain levels sufficient to inhibit <sup>3</sup>H-diazepam binding. Psychopharmacology (in press).
4. Trams, E., Kaufmann, H. and Burnstock, G.: A proposal for the role of ecto-enzymes and adenylates in traumatic shock. J. Theor. Biol. 87: 609-621 1980.
5. Trams, E. G., Lauter, C. J., Salem, N. and Heine, U.: Exfoliation of membrane ecto-enzymes in the form of microvesicles. Biochim. Biophys. Acta (in press).



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER Z01 NS 01808-12 DMN																
PERIOD COVERED October 1, 1980 through September 30, 1981																		
TITLE OF PROJECT (80 characters or less)  Glycoproteins of Myelin in Development and Disease																		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 80%;">PI: R. H. Quarles, Chief, Myelin and Brain Development Section</td> <td style="width: 20%;">DMN NINCDS</td> </tr> <tr> <td>OTHER: R. O. Brady, Chief</td> <td>DMN NINCDS</td> </tr> <tr> <td>D. A. Figlewicz, Guest Worker</td> <td>DMN NINCDS</td> </tr> <tr> <td>D. Johnson, Visiting Fellow</td> <td>DMN, NINCDS</td> </tr> <tr> <td>S. Sato, Visiting Fellow</td> <td>DMN, NINCDS</td> </tr> <tr> <td>T. Inuzuka, Visiting Fellow</td> <td>DMN, NINCDS</td> </tr> <tr> <td>G. Barbarash, Chemist</td> <td>DMN, NINCDS</td> </tr> <tr> <td>T. Neff, Chemist</td> <td>DMN, NINCDS</td> </tr> </table>			PI: R. H. Quarles, Chief, Myelin and Brain Development Section	DMN NINCDS	OTHER: R. O. Brady, Chief	DMN NINCDS	D. A. Figlewicz, Guest Worker	DMN NINCDS	D. Johnson, Visiting Fellow	DMN, NINCDS	S. Sato, Visiting Fellow	DMN, NINCDS	T. Inuzuka, Visiting Fellow	DMN, NINCDS	G. Barbarash, Chemist	DMN, NINCDS	T. Neff, Chemist	DMN, NINCDS
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G. Barbarash, Chemist	DMN, NINCDS																	
T. Neff, Chemist	DMN, NINCDS																	
COOPERATING UNITS (if any) Cellular Neuropathology Section, LNNS, NINCDS Veteran's Administration Hospital, Portland, Oregon																		
LAB/BRANCH Developmental and Metabolic Neurology Branch, NINCDS																		
SECTION Myelin and Brain Development																		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD. 20205																		
TOTAL MANYEARS: 6.9	PROFESSIONAL: 4.1	OTHER: 2.8																
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																		
SUMMARY OF WORK (200 words or less - underline keywords) Central and peripheral <u>myelin</u> sheaths contain a high molecular weight <u>glycoprotein</u> that is selectively localized in the inner portion of the sheaths adjacent to the axon. This glycoprotein, referred to as the <u>myelin-associated glycoprotein (MAG)</u> , is specific for the nervous system and is present in greater amounts in the CNS than in the PNS. A major structural component of compact peripheral myelin (PO) is also glycosylated. Since membrane glycoproteins are known to be involved in recognition and cell-cell interactions, these myelin-related glycoproteins are being studied with regard to their likely roles in <u>glia-axon interactions</u> and myelin compaction. MAG undergoes a decrease in molecular size during <u>development</u> that correlates well with myelin maturation. The chemical and immunological properties of the mature and immature forms of MAG are being investigated. Since glycoproteins are well known to be <u>cell-surface antigens</u> and <u>receptors for viruses</u> , MAG may be involved in the putative autoimmune or viral aspects of <u>multiple sclerosis</u> or other <u>demyelinating diseases</u> . MAG is one of the earliest components to be lost in growing multiple sclerosis plaques, suggesting an important involvement in the pathogenesis of this disease.																		

Project Description:

**Objectives:** To investigate the biochemistry of cells of the nervous system, with particular regard to glycoprotein components and their roles in myelination and demyelination. Other myelin, oligodendrocyte, and Schwann cell proteins and lipids are also examined with the ultimate objective of understanding molecular mechanisms of myelin formation and breakdown. Emphasis is placed on the major myelin-associated glycoprotein (MAG) and its role in glia-axon interactions and in demyelinating diseases such as multiple sclerosis.

**Methods:** Radioactive sugar precursors are used to label CNS and PNS glycoproteins. Myelin and other subcellular fractions are purified differential and gradient centrifugation. Purified myelin is separated into light, medium and heavy subfractions and other oligodendroglia-derived membranes are also isolated. The membrane-bound proteins and glycoproteins are fractionated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. Double label counting techniques are used for detecting radioactive glycoproteins on gels and for revealing differences between samples. Densitometric scanning of gels which have been stained with Coomassie blue for proteins or periodic acid-Schiff reagents for carbohydrate is used for quantitation of individual proteins or glycoproteins. Quantitation of individual lipids is done by thin-layer chromatography and densitometry. Purification of the myelin associated glycoprotein (MAG) and other myelin proteins is achieved by solvent fractionation and gel filtration in the presence of detergents. Peptide mapping is done by partial proteolysis in the presence of SDS followed by separation of peptides on polyacrylamide slabs. Gas liquid chromatography and colorimetric procedures are used for measuring amounts of various carbohydrates. Amino acid compositions are determined on an amino acid analyzer by ninhydrin or fluorometric procedures. Antibodies are prepared in rabbits by conventional techniques and monospecific antibodies are obtained by hybridoma techniques. Antibodies are detected by immunodiffusion, double antibody immune precipitation of radioiodinated antigen, or ELISA procedures. MAG is quantitated by a double antibody, competitive, radioimmunoassay utilizing radioiodinated MAG. Protein antigens that have been separated by SDS slab gel electrophoresis are detected by immune staining after electrotransfer onto nitrocellulose sheets.

**Major Findings:** Research has continued on the isolation and characterization of the major myelin-associated glycoprotein (MAG) with the ultimate objective of understanding its role in the myelin sheath at the molecular level. The purification involving selective extraction of MAG from isolated myelin with lithium diiodosalicylate (LIS)-phenol and gel filtration on Sepharose CL-6B in the presence lithium dodecyl sulfate (LDS) has now become a standard procedure. The column profile at 206 nm is so reproducible that monitoring [<sup>3</sup>H]fucose to detect MAG, as we have done in the past, is not necessary, and it is possible to purify unlabeled MAG from human tissue or other sources. In order to facilitate protein studies on very small amounts of MAG or its fragments, our Beckman 121 MB amino acid analyzer, that is normally used for ninhydrin based analyses, has been modified to do fluorescent analyses with o-phthalaldehyde. This is the first time that this instrument has been used for this methodology, and the modifications are such that the instrument can readily be switched back and forth from o-phthalaldehyde to ninhydrin techniques. Good agreement on the amino acid composition of MAG and other proteins is obtained by the two methods. Rat brain MAG consists of one-third carbohydrate and two-thirds polypeptide. The most abundant amino acids are glycine, serine, and glutamic acid. The overall composition is 20% acidic,

9% basic, and 23% hydrophobic amino acids. The oligosaccharide portion of the molecule on a weight basis is 5% fucose, 23% mannose, 20% galactose, 34% N-acetylglucosamine, and 18% N-acetylneuraminic acid. The larger, immature rat MAG and human MAG are also being purified for chemical and immunological characterization.

The specific antisera that have been prepared to MAG continue to be very useful reagents for a variety of studies. One of the principal uses is in the radioimmunoassay (RIA) for MAG that was described in last year's report. This assay is a very sensitive one and allows us for the first time to measure MAG in whole tissue homogenates and other crude samples. Initial results obtained on whole rat brain during development were also described in last year's report. Studies are in progress on well defined brain regions to compare the developmental appearance of MAG with that of other myelin constituents such as basic protein, proteolipid, and 2'3'-cyclic nucleotide phosphohydrolase. Studies are also underway to compare MAG levels with other myelin constituents in several strains of mutant mice with hypomyelination including Jimmy, Quaking and myelin deficient mice (mld). The RIA was adapted for use on human samples by iodinating purified human MAG and using human MAG as a standard. This was necessary to compensate for the different affinities of the antisera for human and rat MAG. The modified assay gave MAG values of 4.7 and 5.5 ng/ $\mu$ g total protein for human white matter and isolated myelin, respectively. A major effort in the laboratory this year has been to use the radioimmunoassay to measure MAG in multiple sclerosis tissue as described in the next paragraph.

Previous immunocytochemical studies with MAG antisera had indicated that MAG was decreased more than other myelin constituents in the periphery of multiple sclerosis plaques. In order to quantitatively document these findings, experiments were done in which frozen, plaque-containing, multiple sclerosis tissue was obtained from the Human Neurological Specimen Bank at the V.A. Wadsworth Medical Center in California. Samples of macroscopically normal appearing white matter (NAWM), outer periplaque (OPP), inner periplaque (IPP) and plaque (P) were dissected for biochemical analysis. MAG was measured by RIA; basic protein and proteolipid were quantitated by densitometry of SDS slab gels; the activity of 2'3'-cyclic nucleotide phosphohydrolase was measured enzymatically. As expected all of these components were drastically reduced in the plaque where there was nearly complete loss of myelin. However, each constituent was also significantly reduced in OPP and NAWM regions, and the reduction in MAG was consistently greater than the reduction in the other components. For example, in OPP regions MAG was 67% of control levels whereas basic protein was only reduced to 84% of the control value. The greater reduction of MAG was statistically significant. The relatively greater reduction in MAG was most apparent in the most rapidly progressing case of multiple sclerosis that we examined, which is in agreement with the immunocytochemistry which had also indicated that the preferential loss of MAG is greatest in acute, active multiple sclerosis. Immune staining also revealed that MAG decreases prior to basic protein loss in progressive multifocal leukoencephalopathy.

Collaborative immunocytochemical studies on myelin proteins have continued with the Section on Cellular Neuropathology, LNNS. The most important advances this year have involved the peripheral nervous system. Staining for the major



PO glycoprotein of peripheral myelin in open sections of sciatic and trigeminal nerve showed that PO was present in the Golgi membranes of Schwann cells, indicating that the Golgi apparatus is involved in myelinogenesis. Also newly formed myelin sheaths could be detected more readily by immune staining for PO staining than with histological procedures. Myelin breakdown in the PNS was examined in the lesions from patients with idiopathic polyneuritis by staining for PO, P1, and P2 proteins as well as for MAG. All of these myelin sheath proteins decreased simultaneously in this disease, and there did not appear to be an early preferential loss of MAG or any of the other proteins.

Additional immunochemical methodologies have been introduced into the laboratory which should prove very valuable in future studies of myelin proteins in myelinogenesis and demyelination. One of these is the specific immune staining of myelin proteins after they have been separated on SDS slab gels. This involves the electrophoretic transfer of the proteins from the polyacrylamide slabs to nitrocellulose sheets, followed by immunostaining with peroxidase-labeled second antibodies. The technique has been successfully applied to MAG, basic protein, and P2 protein. The procedure was used to show that CNS myelin purified from rabbit spinal cord contains P2 protein, confirming the immunocytochemical indication P2 is a component of CNS myelin of some species. It was also shown that the staining of electrophoretically separated proteins is more sensitive if the immune staining is done with the peroxidase-antiperoxidase (PAP) procedure than with peroxidase-labeled second antibody. Using the PAP technique we can easily detect 5 ng of MAG after electrophoresing as little as 2 µg protein of total rat brain homogenate. We are also progressing with our efforts to produce monoclonal antibodies to MAG. A rapid solid phase, ELISA procedure for screening for anti-MAG antibodies has been developed. Mice have been immunized with both rat and human MAG, and it is hoped that hybridomas secreting monospecific anti-MAG antibodies will be available soon.

The neutral protease activity in purified human and rat myelin that was reported last year has been further characterized. This protease converts MAG to a lower molecular weight derivative (dMAG) that has a molecular weight about 10,000 daltons less than intact MAG, contains about the same amount of carbohydrate as MAG, and reacts with anti-MAG antibodies. The protease also degrades basic protein while other myelin proteins are unaffected. Human MAG is very susceptible to the activity of the protease; 50% is converted to dMAG in a 20 min incubation, whereas 18h is required to degrade 50% of basic protein. Further purification of the myelin by several gradient procedures failed to significantly reduce this proteolytic activity acting on endogenous myelin proteins, suggesting that it may be true myelin-related enzyme that is involved in the natural catabolism of myelin proteins. The high susceptibility of human MAG to its action suggests that it may be involved in myelin breakdown in human demyelinating conditions. This possibility is supported by our finding that MAG and basic protein are affected significantly more rapidly by the protease in myelin isolated from multiple sclerosis brain than in myelin from control human brain. It is of interest that the endogenous protease of human myelin is unique in its very rapid action on MAG, since exogenously added proteases such as trypsin and plasmin effect basic protein much more readily than MAG.

**Significance:** The development of an RIA for MAG was a major breakthrough in the investigation of this glycoprotein, since it is now possible to measure MAG directly in whole tissue samples. This permits the quantitative comparison of MAG levels to those of other myelin constituents, such as basic protein, 2'3'-cyclic nucleotide phosphohydrolase, etc., in normal myelinating tissues, mutant mice exhibiting hypomyelination, and in demyelinating diseases. With regard to myelin formation, there is considerable evidence in the literature indicating that cell surface glycoproteins are involved in recognition and cell-cell interactions. The periaxonal localization of MAG in the CNS suggests that it could be involved in oligodendroglial-axonal interactions during the course of myelinogenesis. Its similar periaxonal localization in the PNS suggests that it may also be involved in Schwann cell-axon interactions. The peptide mapping studies completed in the past year show that the chemistry of the MAG molecule in the PNS is very similar to that in the CNS. Involvement of MAG in a common mechanism of glial-axonal interaction in the two branches of the nervous system is consistent with recent evidence from other laboratories indicating that Schwann cells and oligodendrocytes are capable of myelinating axons from either the PNS or CNS. The higher level of MAG in the CNS may be needed for the more complex interaction of a single oligodendrocyte with many axons in contrast to the simpler one-to-one interaction of Schwann cells with axons in the PNS.

Demyelinating diseases such as multiple sclerosis are believed to involve autoimmune or viral processes. Since membrane glycoproteins are known to be cell surface antigens and receptors for viruses, it is not unlikely that MAG could be involved in the putative autoimmune or viral aspects of multiple sclerosis. The quantitative finding by RIA that MAG is decreased more than other myelin proteins in the periphery of multiple sclerosis plaques confirms and extends the earlier immunocytochemical observation of early MAG loss in the pathology of multiple sclerosis. Since the MAG RIA is done on tissue samples that have been solubilized at 100°C in the detergent, sodium dodecyl sulfate, the decrease in MAG immune reactivity cannot be due to inaccessibility of the antigen. The early loss of MAG may reflect a direct involvement in the putative autoimmune or viral aspects of the disease. An alternative hypothesis is that since the periaxonal MAG is at the most distal location from the oligodendrocyte cell body, its loss may reflect a dying-back pathology in the oligodendrocyte. If so, the fate of MAG in demyelinating situations may provide a means of distinguishing between the myelin-forming cell (oligodendrocyte or Schwann cell) or the myelin sheath itself as the primary target. This hypothesis is supported by immunocytochemical demonstration of the early loss of MAG in progressive multifocal leukoencephalopathy (a known viral infection of the oligodendrocyte) and the absence of early MAG loss in diseases where myelin is probably the primary target such as acute experimental allergic encephalomyelitis (EAE), hexachlorophene intoxication, and idiopathic polyneuritis. According to this hypothesis, the early loss of myelin in multiple sclerosis would suggest that the primary target in this disease is the oligodendrocyte. There is currently a large amount of interest in the role of neutral proteases in demyelinating diseases, and basic protein is generally believed to be the molecule which is most susceptible to these enzymes. Our finding that human MAG is even more susceptible to a neutral protease in isolated myelin may also be very relevant to the biochemistry of demyelination. For these reasons, we think that continuing studies on the chemical and immunological properties of MAG will increase our understanding of the molecular mechanisms underlying demyelination.



Proposed Course: Purification and characterization of MAG both from rat and human sources will continue. Fragments of MAG produced by proteolytic or chemical treatment will be studied with the ultimate objective of determining its overall molecular structure. The chemistry of rat MAG can be related to the mechanism of myelinogenesis which is best studied in this species, whereas the chemistry of human MAG is important for understanding the molecular mechanisms of demyelinating diseases. Peptide mapping studies are in progress to more precisely define the chemical differences between immature and mature MAG. The immunologically active sites in the MAG molecule will be determined. Since the anti-MAG antibodies prepared in rabbits react with sites that are inaccessible in intact membranes, monoclonal antibodies to MAG will be raised with the hope of obtaining antibodies to more accessible portions of the molecule. Antibodies of this type will be more useful for studying the function of the molecule.

The RIA for MAG will be used to compare the deposition of this glycoprotein with other myelin constituents in defined myelinating tracts. Similar studies will be done in mutant mice affected by abnormalities in the mechanism of myelin formation. Tunicamycin and other inhibitors of protein glycosylation will be used in myelinating tissue culture systems (explants or reaggregating cultures) to investigate the role of the sugars in the function of this glycoprotein. Since the periaxonal localization of MAG strongly suggests an involvement in interactions with the axon, attempts will be made to isolate the putative axonal receptor for MAG from fractions enriched in axolemma.

The biochemistry of experimental demyelinating diseases which are believed to affect myelin itself will be compared with that of diseases which affect primarily the oligodendrocyte. Demyelinating agents which may be examined include lysolecithin, ethidium bromide, cuprizone, and diphtheria toxin. The purpose of these experiments will be to see if the nature of the pathology can be correlated with the effect on MAG relative to other myelin proteins. Studies on multiple sclerosis will continue. In particular, the cerebrospinal fluid will be examined for the presence of MAG or its fragments. In addition, multiple sclerosis patients will be tested for humoral or cellular immunity to MAG.

#### Publications:

1. Figlewicz, D. A., Quarles, R. H., Johnson, D., Barbarash, G. R., and Sternberger, N. H.: Biochemical demonstration of the myelin-associated glycoprotein in the peripheral nervous system. J. Neurochem. in press.
2. Quarles, R. H.: Glycoproteins from central and peripheral myelin. In Hashim, G. (Ed.) Myelin: Chemistry and Biology, New York, Alan Liss, 1980, pp 55-77.
3. Quarles, R. H., Johnson, D., Brady R. O. and Sternberger, N. H.: Preparation and characterization of antisera to the myelin-associated glycoprotein. Neurochem. Res. in press.
4. Seil, F. J., Kies, M. W., Agrawal, H. C., Quarles, R. H. and Brady, R. O.: Myelin proteins dissociated from induction of antimyelin antibodies. In Giacobini, E. et. al. (Eds.): Tissue Culture in Neurobiology. Raven Press, New York, 1980, pp. 477-488.

5. Seil, F. J., Quarles, R. H., Johnson, D. and Brady, R. O.: Immunization with purified myelin-associated glycoprotein does not evoke myelination-inhibiting or demyelinating antibodies. Brain Res. 209, 470-475, 1981.
6. Trapp, B. D., Itoyama, Y., Sternberger, N. H., Quarles, R. H. and Webster, H. deF.: Immunocytochemical localization of PO in Golgi membranes and myelin of developing rat Schwann cells. J. Cell. Biol. in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02162-07 DMN
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less) Synthesis of Compounds Analogous to Glycolipids		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: A. E. Gal, Chief, Neurochemical Methodology Section DMN, NINCDS OTHER: F. J. Fash, Bio. Lab. Technician DMN, NINCDS		
COOPERATING UNITS (if any) None		
LAB/BRANCH Developmental and Metabolic Neurology Branch		
SECTION Neurochemical Methodology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.6	PROFESSIONAL: 0.3	OTHER: 0.3
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) <u>Glycolipid analogues</u> of sphingolipids were synthesized that yield a <u>chromo- genic moiety</u> on enzymatic hydrolysis. These compounds are used as reagents for the diagnosis of <u>Niemann-Pick</u> , <u>Gaucher's disease</u> and <u>Krabbe's disease</u> . The chromogenic analogues are also useful for the identification of <u>hetero- zygous carriers</u> of these disorders and for the <u>pre-natal diagnosis</u> of these diseases.		

## Project Description:

Objectives: The compounds to be synthesized in the framework of this project are molecules similar to glycolipids which when cleaved enzymatically provide a chromophore useful for the diagnosis of lipid storage diseases and for the identification of heterozygous carriers.

Methods and Major Findings: Work is underway on the synthesis of a substrate for the chromogenic diagnosis of Farber's disease, a disorder characterized by a deficiency of ceramidase. This compound will be chemically related to 2-hexadecanoylamino-4-nitrophenyl phosphorylcholine (HNP). This substance resembles sphingomyelin but has a benzene ring instead of the aliphatic chain. Due to its nitrophenyl moiety, it yields an intense yellow color upon enzymatic cleavage. It is a reliable chromogenic substrate for assaying sphingomyelinase activity in diverse human tissue samples. It is used for the diagnosis of homozygotes and detection of heterozygous carriers of Niemann-Pick disease. This compound was synthesized by Calbiochem and Koch-Light and is commercially available from these manufacturers. We have developed a simplified synthesis of HNP using phosphorylcholine as the starting material. This improvement was realized because of the availability of free phosphorylcholine for which we developed a practical method of synthesis of HNP using phosphorylcholine as the starting material. This improvement was realized because of the availability of free phosphorylcholine for which we developed a practical method of synthesis. Based on the chemistry of HNP, research on non-radioactive sphingolipid substrates was extended to other lipidoses. Compounds were synthesized which could be used as substrates for measuring gluco- and galactocerebrosidase activities in tissue extracts. Thus, 2-hexadecanoylamino-4-nitrophenyl glucoside HNGlu was shown to be a useful compound for the diagnosis of Gaucher's disease and 2-hexadecanoylamino-4-nitrophenyl galactoside HNGal can be used for the diagnosis of Krabbe's disease. These three compounds are now commercially available from several chemical supply companies.

Significance: The new compounds were thoroughly tested and they have been found to be reliable for the diagnosis of lipid storage diseases. These findings constitute a major breakthrough because the previously required radiolabeled products are scarce, expensive, and not widely available. The chromogenic substances can be used and easily handled by practitioners and clinical chemists with no danger of radioactive contamination and they eliminate the necessity of costly and complex radioactive detection techniques.

Proposed Course: Based on the concept demonstrated by this project, additional compounds will be synthesized with chromophoric moieties for the detection of other enzyme deficiency disorders.

## Publications:

None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02163-07 DMN				
PERIOD COVERED October 1, 1980 through September 30, 1981						
TITLE OF PROJECT (80 characters or less) Development of Special Analytical Methods and Preparative Techniques to Investigate the Etiology and Therapy of the Sphingolipidoses						
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%;"> <tr> <td style="width: 70%;">PI: A. E. Gal, Chief, Neurochemical Methodology Section</td> <td style="width: 30%;">DMN NINCDS</td> </tr> <tr> <td>OTHER: F. J. Fash, Biol. Lab. Technician</td> <td>DMN NINCDS</td> </tr> </table>			PI: A. E. Gal, Chief, Neurochemical Methodology Section	DMN NINCDS	OTHER: F. J. Fash, Biol. Lab. Technician	DMN NINCDS
PI: A. E. Gal, Chief, Neurochemical Methodology Section	DMN NINCDS					
OTHER: F. J. Fash, Biol. Lab. Technician	DMN NINCDS					
COOPERATING UNITS (if any) None						
LAB/BRANCH Developmental and Metabolic Neurology Branch						
SECTION Neurochemical Methodology Section						
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205						
TOTAL MANYEARS: <div style="text-align: center;">0.8</div>	PROFESSIONAL: <div style="text-align: center;">0.4</div>	OTHER: <div style="text-align: center;">0.4</div>				
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;"> <input type="checkbox"/> (a) HUMAN SUBJECTS         </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (b) HUMAN TISSUES         </div> <div style="width: 30%;"> <input type="checkbox"/> (c) NEITHER         </div> </div> <div style="display: flex; justify-content: space-between; align-items: flex-start; margin-top: 5px;"> <div style="width: 30%;"> <input type="checkbox"/> (a1) MINORS         </div> <div style="width: 30%;"> <input type="checkbox"/> (a2) INTERVIEWS         </div> </div>						
SUMMARY OF WORK (200 words or less - underline keywords) <p> <u>New analytical techniques</u> were developed and used in enzymatic research and in clinical investigations of <u>lipidoses</u>. The lipid content in human tissues, the diagnosis of lipid storage diseases by <u>gas, thin-layer chromatography</u> and other techniques were studied at the microgram level. The techniques we developed previously were improved, modified and used in connection with ongoing projects related to lipidoses in our laboratories and also as joint projects with outside groups.         </p>						



Project Description:

Objectives: To develop techniques by which the separation and chemical analysis of biologic materials related to sphingolipidoses can be advanced. This work involves the following approaches: 1. Improvement of techniques leading to the separation of enzymes. 2. Development of ultramicro analytical methods for the determination of lipids in biological materials.

Methods: The development of methods for the determination of lipids in small samples of biological materials of human origin such as erythrocytes, leukocytes, fibroblasts, serum, cerebrospinal fluid, urine or biopsy samples from kidney, liver and brain. The individual sphingolipids are present usually only in submicrogram quantities in these samples. For the separation of such lipids, thin layer and gas chromatographic procedures combined with column-liquid chromatography was used.

Quantitative evaluation was made by scanning of the thin-layer plates or by gas chromatography. Much work was done in areas not covered by existing literature references.

Major Findings: Gas chromatography of glucose originating from lipids could not be determined previously. This problem was solved by us. Also a new thin-layer chromatography system was developed which resulted in more reliable results using only small amounts of specimen. A novel technique was developed in which lipids present in the same sample (but not attacked by the exogenous enzyme) were used as internal standards. Improved analytical techniques showed practical results particularly in the studies related to replacement therapy of enzymes where the decrease of lipid levels in the liver and erythrocytes of patients was established and through these procedures an evaluation of the therapeutic effect of enzyme administration can be assessed.

Significance: The purification of the missing enzymes required for the therapy of the lipid storage diseases is a complex, tedious, and costly procedure. The identification of accumulated lipids in human tissues for the diagnosis and control of inherited lipid diseases is dependent on the sensitivity of the analytical techniques. The importance of accuracy in working with trace amounts of material in biological specimens necessitates improved techniques at the submicrogram level.

Proposed Course: Much more work has to be done in relation to the improvement of microanalytical procedures; for example, the ultramicrodetermination of aminosugars and sialic acid needs further development. Some of the existing methods are too complex and their simplification will be investigated. The application of other techniques including high speed (or pressure) liquid chromatography or the use of mass spectroscopy will be explored.

Publications:

Pentchev, P..G., Gal, A. E., Wong, R., Morrone, S., Neumeyer, B., Massey, J., Kanter, R., Sawitsky, A., and Brady, R. O. Biliary excretion of glycolipid in induced or inherited glycosylceramide lipidosis. Biochim. Biophys. Acta 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02366-03 DMN						
PERIOD COVERED October 1, 1980 through September 30, 1981								
TITLE OF PROJECT (80 characters or less) Regulation of Hormone-Responsive Adenylate Cyclase								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%;"> <tr> <td style="width: 60%;">PI: P. H. Fishman, Chief, Membrane Biochemistry</td> <td style="width: 40%;">DMN, NINCDS</td> </tr> <tr> <td>OTHER: J. Hagmann, M.D., Visiting Associate</td> <td>DMN, NINCDS</td> </tr> <tr> <td>S. Kassiss, Ph.D., Visiting Fellow</td> <td>DMN, NINCDS</td> </tr> </table>			PI: P. H. Fishman, Chief, Membrane Biochemistry	DMN, NINCDS	OTHER: J. Hagmann, M.D., Visiting Associate	DMN, NINCDS	S. Kassiss, Ph.D., Visiting Fellow	DMN, NINCDS
PI: P. H. Fishman, Chief, Membrane Biochemistry	DMN, NINCDS							
OTHER: J. Hagmann, M.D., Visiting Associate	DMN, NINCDS							
S. Kassiss, Ph.D., Visiting Fellow	DMN, NINCDS							
COOPERATING UNITS (if any) Laboratory of Molecular Biology, NINCDS Biological Psychiatry Branch, NIMH								
LAB/BRANCH Developmental & Metabolic Neurology Branch								
SECTION Membrane Biochemistry								
INSTITUTE AND LOCATION NINCDS, NIH Bethesda, Md. 20205								
TOTAL MANYEARS: 2.6	PROFESSIONAL: 2.0	OTHER: 0.6						
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS								
SUMMARY OF WORK (200 words or less - underline keywords) 1. Prolonged exposure of human fibroblasts to PGE <sub>1</sub> results in a <u>desensitization of adenylate cyclase</u> . The enzyme exhibits a reduced response to not only PGE <sub>1</sub> , but to other effectors including <u>cholera toxin</u> . When membranes from control and desensitized cells are incubated with activated cholera toxin and [ <sup>32</sup> P] NAD, however, the toxin catalyzes the ADP-ribosylation of the <u>guanine nucleotide regulatory component</u> of the cyclase equally well in both membranes. When adenylate cyclase is solubilized and analyzed by sucrose density gradient centrifugation, the activity from PGE <sub>1</sub> -treated cells has an apparent lower molecular weight than the enzyme from control cells. PGE <sub>1</sub> -mediated desensitization may involve an uncoupling of the regulatory and catalytic components of adenylate cyclase. 2. In intact cells, cholera toxin activates adenylate cyclase after a lag period. Using an immunochemical procedure, bound toxin appears to be internalized during the lag period. In contrast, there is no degradation of the toxin during the lag and activation periods. Generation of the A <sub>1</sub> subunit of the toxin parallels the activation of adenylate cyclase. Thus, intact cells first internalize cholera toxin and reduce it to form A <sub>1</sub> , which can activate adenylate cyclase.								

Project Description:

Objectives: To investigate the molecular mechanisms involved in the regulation of hormone-responsive adenylate cyclase systems; to examine hormone-responsive adenylate cyclase during development and differentiation; to develop an overall model for this example of transmembrane signalling; to relate these findings to various metabolic disorders.

Methods: Neuroblastoma (clone NB), Friend erythroleukemic, HeLa and rat glial C6 cells are established cell lines. Human fibroblasts are obtained from skin biopsies of normal donors. Cells grown in monolayer culture on plastic dishes or in suspension are incubated with hormones and other effectors and levels of cyclic AMP are measured with a modified cyclic AMP protein binding assay. Adenylate cyclase activity in cell lysates is determined in the presence and absence of hormones and other effectors. Binding of hormones and toxins to intact cells and cell membranes is measured with radioactively labeled ligands. Bound ligand is separated from free ligand by rapidly washing the monolayer cultures or by filtering the cells and membranes on small filters by means of a vacuum manifold. Specific binding is determined by correcting for radioligand bound in the presence of excess unlabeled ligand. Membranes are extracted with detergents and the soluble components analyzed by sucrose density gradient centrifugation. Membrane components are ADP-ribosylated by incubating them with activated cholera toxin and [ $^{32}$ P] NAD and separating them by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Major Findings:A. Mechanisms of Hormone-Mediated Desensitization

Prolonged exposure of human fibroblasts to the beta-adrenergic agonist isoproterenol (ISO) or to prostaglandin $E_1$  (PGE $_1$ ) results in a loss of hormone-stimulated adenylate cyclase activity. Desensitization to ISO appears to be specific as the cyclase remains responsive to PGE $_1$ , NaF and cholera toxin (CT). In contrast, the PGE $_1$ -desensitized cells exhibit less cyclase activity to ISO, NaF and CT. Both ISO- and PGE $_1$ -treated cells retain their beta-adrenergic and CT receptors. When membranes are incubated with the A $_1$  subunit of CT and [ $^{32}$ P]-NAD and analyzed by SDS-PAGE, two proteins are specifically ADP-ribosylated by the toxin. Their respective molecular weights are 42,000 and 47,000. Numerous studies by others indicate that these two proteins are components of the guanine nucleotide regulatory component of the adenylate cyclase system and that ADP-ribosylation is the mechanism by which the toxin persistently activates the cyclase. Labeling of these two proteins was not reduced in either ISO- or PGE $_1$ -desensitized cells. When membranes are extracted with Triton and the solubilized adenylate cyclase activity analyzed on sucrose density gradients, the enzyme from PGE $_1$ -desensitized cells has a lower apparent molecular weight than that from control cells. Based on these results, we believe that desensitization does not involve a loss of the receptor, regulatory or catalytic components of adenylate cyclase but rather uncoupling of these components. In the case of beta-agonist mediated desensitization, there is an uncoupling between the receptor and the regulatory component and in the case of PGE $_1$ -mediated desensitization, between the regulatory and catalytic components.

## B. Further Studies on the Mechanism of Action of Cholera Toxin

It is generally accepted that CT binds to the ganglioside  $G_{M1}$  on the cell surface through its B component and that the  $A_1$  subunit, which is an ADP-ribosyltransferase, catalyzes the transfer of ADP-ribose from NAD to the guanine nucleotide regulatory component of adenylate cyclase. This modification results in a persistently activated form of the cyclase. The intervening membrane events between binding and activation have not been elucidated. Using antibodies to CT and  $^{125}\text{I}$ -labeled Protein A, we developed a highly specific and sensitive assay for CT bound to the cell surface. When NB cells were incubated with CT at  $4^\circ\text{C}$ , washed and shifted to  $37^\circ\text{C}$  in fresh medium, the ability of the cells to bind antitoxin and Protein A decreased rapidly and preceded the activation of adenylate cyclase. The latter process is initiated after a 15 min lag and is complete by 45 to 60 min. When  $^{125}\text{I}$ -CT was used, there was no evidence of degradation of CT until after 45 min at  $37^\circ\text{C}$ . All three processes were blocked in cells maintained at  $40^\circ\text{C}$ . At  $22^\circ\text{C}$ , internalization and activation occurred at a slower rate whereas degradation was almost completely inhibited.

Cells were incubated at  $4^\circ\text{C}$  with  $^{125}\text{I}$ -CT, washed and shifted to  $37^\circ\text{C}$  for increasing times. The cells were then lysed, dissolved in SDS and analyzed by SDS-PAGE. Between 0 and 15 min at  $37^\circ\text{C}$ , only a trace amount (less than 0.5%) of the radioactivity corresponded to the  $A_1$  subunit of CT. Then the appearance of  $A_1$  increased in parallel with the activation of adenylate cyclase and reached 5 to 6% of the total radioactivity. Generation of  $A_1$  by intact cells was temperature sensitive and corresponded to cyclase activation. Addition of antitoxin to the cells prior to the shift to  $37^\circ\text{C}$  blocked both the generation of  $A_1$  and the activation of cyclase. When the lysed cells were separated into membranes and cytosol, more of the  $A_1$  was found in the cytosol than in the membranes. When cells were incubated at  $15^\circ\text{C}$  with CT, there was no activation of cyclase. When the cells were incubated at  $37^\circ\text{C}$  with CT for 20 min (to partially activate the cyclase) and then shifted to  $15^\circ\text{C}$ , cyclase continued to be activated albeit at a slower rate than that observed at  $37^\circ\text{C}$ .

We conclude from these studies that after CT binds to the cell surface, it or some portion of it penetrates the membrane. Once across the membrane, the  $A_1$  subunit is released into the cytosol and can activate adenylate cyclase. Later,  $A_1$  becomes degraded presumably in the lysosomes.

Significance: These studies are providing information on the molecular mechanisms involved in regulating adenylate cyclase activity. Hormone-mediated desensitization of adenylate cyclase may be homologous or heterologous. In either case, there appears to be no loss of various components of the adenylate cyclase system but an uncoupling of these components. Some of the intervening events between the binding of CT and its action have finally been clarified. An essential step is the generation of  $A_1$ , the active component of the toxin, in the intoxicated cell.

Proposed Course: The project will be continued. Attempts will be made to determine the biochemical mechanism involved in the uncoupling of adenylate cyclase components during desensitization.



Publications:

1. Fishman, P. H., Mallorga, P. and Tallman, J.F.: Catecholamine-induced desensitization of adenylate cyclase in rat glioma C6 cells: evidence for a specific uncoupling of  $\beta$ -adrenergic receptors from a functional regulatory component of adenylate cyclase. Mol. Pharmacol., in press.
2. Hagmann, J. and Fishman, P. H.: Inhibitors of protein synthesis block action of cholera toxin. Biochem. Biophys. Res. Commun. 98: 677-684, 1981.
3. Parent, J. B., Tallman, J. F., Henneberry, R. C. and Fishman, P. H.: Appearance of  $\beta$ -adrenergic receptors and catecholamine-responsive adenylate cyclase during fusion of avian embryonic muscle cells. J. Biol. Chem. 255: 7782-7786, 1980.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> <b>INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 NS 02433-02 DMN
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less) Models of Lysosomal Storage Disease.		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: John A. Barranger, M.D., Ph.D. Other: George Constantopoulos, Ph.D. Igal Gery, Ph.D. F. Scott Furbish, Ph.D. Peggy Rands Susan H. Sorrell Daniela Seminara, Ph.D. Edward I. Ginns, M.D., Ph.D. Norman Barton, M.D., Ph.D. Roscoe O. Brady, M.D.	Chief, Clinical Investigations and Therapeutics Section Research Biochemist Visiting Scientist Staff Fellow Guest Worker Chemist Visiting Fellow Clinical Associate Clinical Associate Chief	DMN NINCDS DMN NINCDS LVR NEI DMN NINCDS DMN NINCDS DMN NINCDS DMN NINCDS DMN NINCDS DMN NINCDS DMN NINCDS
COOPERATING UNITS (if any)  Laboratory of Vision Research, NEI		
LAB/BRANCH Developmental and Metabolic Neurology Branch		
SECTION Clinical Investigations and Therapeutics		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:  2.5	PROFESSIONAL:  2.0	OTHER:  0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The possibility of enzyme replacement in <u>lysosomal storage diseases</u> requires extensive knowledge of the physiology and biochemistry of cells and their organelles, especially, the <u>lysosome</u> in the condition of storage. Animals treated with drugs ( <u>suramin</u> , <u>chloroquine</u> ) simulated lysosomal storage disease. Study of physiologic and biochemical aberrations in treated animals will define the milieu in which enzyme replacement can be attempted. Of particular interest is the ability of treated animals to incorporate infused enzymes, the function of <u>receptors</u> on plasma and lysosomal membranes, the ability of incorporated enzymes to <u>interact</u> with storage material, and the targeting of enzyme to cells and prolongation of their biologic activity <u>in situ</u> . Physiologic alterations are appropriately studied in animals, whereas, more detailed biochemical studies are more suitably conducted in strictly defined cell suspensions and cell cultures. Studies employing animal cells including <u>macrophages</u> , <u>fibroblasts</u> , <u>hepatocytes</u> , and <u>Kupffer cells</u> will be performed. Also, cultures of human fibroblasts and peripheral macrophages from control and disease states will be utilized.		

45 - DMNB/IRP

Project Description:

Objectives: 1) Develop convenient animal models of lysosomal storage; 2) Evaluate morphologic and biochemical changes in treated animals including assay of lysosomal enzymes that are affected and accumulation of substances in lysosomes; 3) Study clearance of infused enzymes from circulation of treated animals; 4) Study incorporation and distribution of infused enzymes into organs of treated animals; 5) Study location, types, and numbers of receptors for lysosomal enzymes in treated animals compared to controls; 6) Evaluate efficiency of interaction of infused enzymes with lysosomal elements; 7) Increase delivery of enzymes to specific cell type and prolong biologic activity; 8) Study effects of complex glycolipids on normal and disease-state cells in culture; 9) Study receptors for glycolipids on membranes of cells in culture; 10) Evaluate effects of coculture of storage cells (e.g., Kupffer cells) with parenchymal cells (e.g., hepatocytes) to appraise interaction between cells; 11) Study location, types, and numbers of receptors for lysosomal enzymes on control and disease-state cells in culture; 12) Study specificity of the receptor and its regulation; 13) Study effects of supplemental enzymes on storage of complex glycolipids by cells in culture and evaluate reversal of changes in those cells; 14) Study the regulation of enzyme content of cells in culture.

Methods Employed:

- 1) Treatment of rats with suramin and chloroquine by injection or oral route.
- 2) Light and electron microscopic examination of tissues.
- 3) Assay of lysosomal enzymes in tissue extracts and lysosomal fractions from treated animals.
- 4) Assays of mucopolysaccharides, glycolipids, and gangliosides in tissues and lysosomal fractions from treated rats.
- 5) Preparation of rats for clearance studies.
- 6) <sup>125</sup>I-labelling of lysosomal enzymes.
- 7) Isolation of purified fractions of rat hepatocytes and Kupffer cells and monolayer culture of these cells.
- 8) Radio-autography of tissues from enzyme supplemented animals and cells in culture.
- 9) Monolayer and suspension cultures of murine and human peripheral macrophages.
- 10) Radioligand-receptor analysis of glycoprotein enzyme receptors and glycolipid receptors of cells in suspension and culture.
- 11) Assay of lymphocyte activating factor, cytoplasmic and lysosomal enzymes released by cells in culture.

Major Findings:

Suramin treated animals demonstrate morphologic similarities to lysosomal storage disorders. Mucopolysaccharides accumulate in tissues of these animals. Gangliosides also accumulated in tissues of suramin treated animals in a pattern similar to the mucopolysaccharidoses. Certain lysosomal enzymes are non-competitively inhibited by suramin *in vitro*. Thus, suramin treated animals are a convenient model of mucopolysaccharidoses.

Non-elicited (resident) murine macrophages in culture accumulate glucosylceramide (GL<sub>1</sub>). Other cells in culture (elicited macrophages, fibroblasts, and lens epithelial cells) do not incorporate significant amounts of the glycolipid. Other complex lipids (ganglioside GM<sub>2</sub>, ceramide trihexoside, and sphingomyelin) are not incorporated significantly. Macrophages (M $\phi$ ) that have accumulated GL<sub>1</sub> increase the production and release of lymphocyte activating factor (LAF) lysosomal enzymes, and cytoplasmic enzymes. The degree of these responses are directly related to the amount of lipid accumulated. Non-virus transformed permanent cell lines of human M $\phi$  from both normal and Gaucher patients have been established. The enzyme content of these cells changes with culture conditions and time.

#### Significance to Bio-Medical Research and the Program of the Institute:

Inborn errors of metabolism including the lysosomal storage disorders are severely disabling or fatal diseases. Most have profound neurologic consequences. The studies conducted in this project are designed to better define the physiology and biochemistry of cells involved in the storage process. An understanding of the pathogenesis and alteration of cell function may assist in the intervention in the disease process with such techniques as enzyme replacement. As an example, the finding that of a number of cells, only macrophages (M $\phi$ ) accumulate GL<sub>1</sub> and release lymphocyte activating factor, may be useful in monitoring the effects of enzyme supplemented to cells. More basically, the peculiar distribution of GL<sub>1</sub> in Gaucher's disease (i.e., only in M $\phi$ ) may be the result of some special or specific property of the cell or glycolipid. Definition of that property may allow its manipulation to permit some measure of control of the disease. The observations that M $\phi$  release activators and lysosomal enzymes in response to storage of GL<sub>1</sub> gives a clue to understanding the multiple clinical problems of Gaucher patients. Particularly interesting is the injury of cells that are not involved in the storage of the lipid itself, but are obviously affected (e.g., parenchyma of liver, bone, and brain). This result may be mediated by the release of lysosomal enzymes and other toxins from M $\phi$ . Release of lymphocyte activating factor may explain the appearance of monoclonal and polyclonal gammopathies in these patients. Moreover, this immunological link could have bearing on the increased risk of malignancy in these patients. The finding concerning the difference between "resident" and "activated" M $\phi$  in their interaction with GL<sub>1</sub> provides a new parameter for defining the changes which accompany M $\phi$  activation. In addition to their role in defense mechanisms against infection or malignancy, the activated M $\phi$  are a major component of inflammation (particularly of the chronic type). These studies could provide a new approach to the characterization of the activated M $\phi$ . The availability and appropriateness of animal and cell models of storage disease is obvious. Studies which cannot be done in humans because of risk or inability to control the variables can be accomplished in these systems. Basic questions relating to the efficiency of enzyme replacement can be answered in the laboratory and the results used to tailor appropriate clinical trials. This will be especially true in studies of human disease macrophages.

Proposed Course of the Project: Areas that will be further investigated will concentrate on the human Gaucher macrophage (M $\phi$ ) in culture. Their ability to incorporate GL<sub>1</sub> will be evaluated particularly with respect to the specificity of uptake. Radio-ligand receptor analysis and autoradiographic techniques will be employed to determine if the monosaccharide of the glycolipid is responsible for the specificity. These techniques will also be employed to examine receptors on these cells and their subcellular elements for lysosomal enzymes. The aim of these

studies is to determine the best possible ligand for the most efficient delivery and longest survival of supplemented enzymes in these cells. It is expected that maximizing these parameters will increase the efficiency of supplemented enzymes in reducing the accumulation of GL<sub>1</sub> and reversing the changes in M $\phi$  produced by the lipid accumulation.

The effects of corticosteroids on human Gaucher macrophages in culture will be evaluated. Parameters to be measured will be changes in endogenous enzyme concentration, changes in receptor-ligand binding, changes in glycolipid availability to supplemented enzymes and efficiency of supplemented enzyme to reduce GL<sub>1</sub> accumulation.

Other objectives to be pursued are the effects of coculture of glycolipid loaded murine Kupffer cells and hepatocytes.

#### Publications:

1. Constantopoulos, G., Rees, S., Cragg, B. G., Barranger, J. A. and Brady, R. O.: Effect of suramin on the activities of degradative enzymes of sphingolipids in rats. Res. Commun. Chem. Path. Pharmacol., 32: 87-97, 1981.
2. Gery, I., Seminara, D., Derr, J. and Barranger, J. A.: Production and release of lymphocyte activating factor (Interleukin 1) by human monocytes and their derived macrophages. In Kirchner, H. and Resch, K. (Eds.): Mechanism of Lymphocyte Activation. Elsevier/North Holland, 1981, in press.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02434-02 DMN																								
PERIOD COVERED <p style="text-align: center;">October 1, 1980 through September 30, 1981</p>																										
TITLE OF PROJECT (80 characters or less) <p>Studies of Lysosomal Function: Receptor-Mediated Pinocytosis of Lysosomal Enzymes.</p>																										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																										
<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 60%;">John A. Barranger, M.D., Ph.D. Chief, Clinical Investigations and Therapeutics Section</td> <td style="width: 10%;">DMN</td> <td style="width: 20%;">NINCDS</td> </tr> <tr> <td rowspan="5">Other:</td> <td>F. Scott Furbish, Ph.D. Staff Fellow</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td>Edward I. Ginns, M.D., Ph.D. Clinical Associate</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td>Norman Barton, M.D., Ph.D. Clinical Associate</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td>Susan H. Sorrell Chemist</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td>Peggy Rands Guest Worker</td> <td>DMN</td> <td>NINCDS</td> </tr> <tr> <td></td> <td>Roscoe O. Brady, M.D. Chief</td> <td>DMN</td> <td>NINCDS</td> </tr> </table>			PI:	John A. Barranger, M.D., Ph.D. Chief, Clinical Investigations and Therapeutics Section	DMN	NINCDS	Other:	F. Scott Furbish, Ph.D. Staff Fellow	DMN	NINCDS	Edward I. Ginns, M.D., Ph.D. Clinical Associate	DMN	NINCDS	Norman Barton, M.D., Ph.D. Clinical Associate	DMN	NINCDS	Susan H. Sorrell Chemist	DMN	NINCDS	Peggy Rands Guest Worker	DMN	NINCDS		Roscoe O. Brady, M.D. Chief	DMN	NINCDS
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SUMMARY OF WORK (200 words or less - underline keywords) <p>The uptake of active <u>glycoprotein lysosomal enzymes</u> occurs, in part, through the mechanism of <u>adsorptive pinocytosis</u>. <u>Receptors</u> for various parts of the enzyme molecule as <u>ligands</u> are present on the <u>plasma and organelle membranes</u>. It is the purpose of this project to study these receptors and utilize them for <u>targeting enzymes</u> to cells. These <u>binding capacities</u> may also play a role in <u>localizing glycoproteins</u> within the <u>cell</u> and thus may have a bearing on the <u>survival of enzymes</u> that have been incorporated into the cell. Thus, these studies are also directed toward increasing the <u>survival of exogenous enzymes</u> within certain <u>subcellular organelles</u>. The goal of these studies is to increase the <u>interaction of exogenous enzyme with stored material</u> in the cell and thereby increase the efficiency of <u>enzyme replacement</u>. These studies will be carried out initially in rats and in later phases in <u>human storage disease macrophages in culture</u>.</p>																										



Project Description:

**Objectives:** 1) Study the clearance of lysosomal enzymes from rat circulation using enzyme activity and  $^{125}\text{I}$ -labelled enzymes; 2) Study the incorporation of infused enzymes and measure distribution to organs of the rat; 3) Study the distribution of infused enzymes to purified preparations of rat liver parenchymal and Kupffer cells; 4) Study survival time of infused lysosomal enzymes in rat liver and isolated hepatocytes and Kupffer cells; 5) Study survival time of infused enzymes in lysosomes of rat liver parenchymal and Kupffer cells; 6) Identify receptors on rat liver parenchymal and Kupffer cells for lysosomal enzymes; 7) Identify receptors on lysosomal and other cellular organelle membranes for lysosomal enzymes; 8) Modify lysosomal enzymes to increase delivery to either hepatocytes or Kupffer cells; 9) Use carbohydrate-enzyme conjugates to target lysosomal enzymes to either hepatocytes or Kupffer cells; 10) Modify lysosomal enzymes to increase survival in rat liver hepatocytes and Kupffer cell lysosomes; 11) Isolate and purify rat liver receptors for lysosomal enzymes; 12) Estimate the effect of steroid treatment on the number and types of receptors in rat liver; 13) Study uptake, survival and receptors for lysosomal enzymes in storage disease macrophages in culture; 14) Study effects of supplemental enzymes on reversing substrate accumulation and secondary alterations in storage disease macrophages; 15) Study efficiency of modified lysosomal enzymes in reversing substrate accumulation in storage disease macrophages; 16) Estimate effects of steroids on efficiency of lysosomal enzymes to reverse substrate accumulation in storage disease macrophages; 17) Study receptors on storage disease macrophages for substrates that are stored; 18) Estimate effect of steroids on the accumulation of substrate in storage disease macrophages.

Methods Employed:

1.  $^{125}\text{I}$ -labelling of lysosomal enzymes.
2. Radioligand-receptor analysis of glycoprotein enzyme receptors and glycolipid receptors on cells in suspension and culture.
3. Monolayer culture of human storage disease macrophages.
4. Radio-autography of cells and organelles.
5. Affinity chromatography of receptors for lysosomal enzymes.
6. Cyanoborohydride, carbodiimide, and other carbohydrate to protein conjugations.
7. Glycosidase modification of lysosomal enzymes.
8. Isolation of purified rat liver hepatocytes and Kupffer cells.
9. Isolation of purified normal and diseased human monocytes.

Major Findings:

Lysosomal glucocerebrosidase is cleared from the circulation in a biphasic manner. The majority of the enzyme is cleared with a half-time of approximately 15 minutes. A smaller amount has a considerably longer half-time in the circulation. The more rapidly cleared form is subject to alteration of its clearance time by glycosidase treatment. The slowly cleared form is not. Approximately 50% of the dose of enzyme appears in the liver. The survival time in liver is approximately 8 hrs. Of the enzyme recovered in liver, most of it is found in hepatocytes. Only about 2% can be recovered in non-parenchymal cells. Sequential treatment of glucocerebrosidase with glycosidases result in the exposure of galactose, N-acetyl-

glucosamine, and mannose moieties such that the monosaccharide is the terminal sugar of the glycoprotein and mediates its uptake. The uptake is saturable and can be competed with by specific monosaccharide terminal glycoproteins. Receptors have been identified on hepatocytes for galactose terminal glucocerebrosidase and on non-parenchymal cells for N-acetylglucosamine and mannose terminal enzyme. Utilization of the receptor on non-parenchymal cells for mannose terminal enzyme permits a 45-fold increase in activity of these cells whereas unmodified enzyme results in only a 7-fold increase in enzyme activity in these cells. Fucose also plays a role in the uptake of glucocerebrosidase in hepatocytes. Treatment of the enzyme with fucosidase results in a significant decrease in uptake. No significant effect is seen on nonparenchymal cell uptake by fucosidase treatment. Studies of hexosaminidase indicate that the lysosomal survival time is identical in hepatocytes and non-parenchymal cells. However, the survival time is considerably longer than for glucocerebrosidase. Analysis of the properties of hexosaminidase that impart this longer survival may allow modification of glucocerebrosidase to increase its survival in lysosomes.

#### Significance to Bio-Medical Research and the Program of the Institute:

The lysosomal storage disorders are disabling or fatal diseases which frequently affect the nervous system. At present, there is no effective treatment for most of these diseases. The idea of enzyme replacement has been proposed and is promising. The studies performed in this project support the concept and confirm earlier observations that enzyme replacement is a possibility. Present investigations are aimed at increasing the delivery of enzymes to cells and maximizing their catabolic activity on stored substrates. Receptors for glucocerebrosidase can be utilized to increase delivery to Kupffer cells. The potential for targeting even greater amounts of enzyme may have been uncovered by these observations. This is fortunate as the Kupffer cells and other similar reticuloendothelial elements are the only cells in Gaucher's disease in which the storage material, glucosylceramide, is found. The results obtained with hexosaminidase may provide useful information which will lead to extending the survival time of other enzymes, such as glucocerebrosidase. Studies of human macrophages obtained from patients with storage disease should facilitate a number of observations in an experimental milieu which can be well controlled and should be pertinent to the actual disease. Results of these experiments should permit tailoring of enzyme for clinical replacement trials.

Proposed Course of the Project: Future work will concentrate on characterizing receptors on the plasma and organelle membranes of various cells, particularly the human macrophage in culture. Methods will be explored to increase interaction of supplemented enzymes with storage material.

Other modalities for increasing enzyme uptake and efficiency such as taking advantage of natural properties of the molecule (e.g., hydrobicity and charge) will be examined. This will necessitate elucidation of the carbohydrate and amino acid composition of the molecules and making appropriate comparisons between molecules that behave differently at the surface of plasma and organelle membranes.

Publications:

1. Furbish, F. S., Krett, N. L., Barranger, J. A. and Brady, R. O.:  
Fucose plays a role in the clearance and uptake of glucocerebrosidase  
by rat liver cells. Biochem. Biophys. Res. Commun., 95: 1768-1774,  
1980.
2. Brady, R. O. and Furbish, F. S.: Enzyme replacement therapy:  
specific targeting of exogenous enzymes to storage cells.  
Membranes and Transport, 1981, in press.
3. Brady, R. O., Barranger, J. A., Pentchev, P. G., Furbish, F. S.  
and Gal, A. E.: Prospects for enzyme replacement therapy in  
heritable metabolic disorders. In Aebi, H. and Herschkowitz, N. N.  
(Eds.): Inborn Errors of Metabolism in Humans. MTP Press, London,  
in press.

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PERIOD COVERED <div style="text-align: center;">October 1, 1980 through September 30, 1981</div>																						
TITLE OF PROJECT (80 characters or less) Studies On The Mechanism Of Pathogenesis Of The Mucopolysaccharidoses.																						
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SUMMARY OF WORK (200 words or less - underline keywords) Objective of this project is the study of <u>mechanism of pathogenesis of the mucopolysaccharidoses (MPS)</u> with emphasis on <u>brain involvement and mental retardation</u> . We are using a <u>comparative approach</u> . For this purpose, the <u>biochemical changes in glycosaminoglycans (GAG), sphingolipids and pertinent lysosomal enzymes</u> are determined in tissues from patients with various types of MPS and are compared with normal controls, and correlations are made in terms of clinical and ultrastructural findings. To complement the studies with human subjects, an <u>animal model of mucopolysaccharidosis</u> is being developed. The drug <u>suramin</u> given intravenously and intracerebrally to rats <u>inhibits enzymes of glycosaminoglycan and sphingolipid degradation</u> and causes <u>accumulation of these compounds in the lysosomes</u> , thus simulating a <u>mucopolysaccharidosi</u> s.																						

Project Description:

Objectives: The mucopolysaccharidoses, like all the other heritable disorders, can be considered experiments of nature. Because of the ubiquitous distribution of glycosaminoglycans in the human body, the lack of enzyme(s) required for their degradation affects many organs and functions. Our objective was to study the mechanism of pathogenesis of the various abnormalities characterizing the patients with MPS, with emphasis on the mechanism of brain involvement. For this purpose we try to correlate the chemical structure(s) of the involved compounds with the observed dysfunction (function). Because of the limitations arising from the lack of human material and of the requirement to modify "the experiment" at will, we are also trying to develop an animal model of MPS. We have administered the trypanocidal drug suramin, intravenously and intracerebrally, to rats. It seems that the suramin-treated rat may provide a useful animal model for the study of MPS.

Patient Material: Tissues were obtained at autopsy from patients with MPS and from several non-neurological patients.

Hooded rats of different ages were used for the development of the experimental model.

Methods Employed:

- 1) Tissues were obtained at autopsy and the white and gray matter and the leptomeninges were carefully dissected.
- 2) In hooded rats, suramin (250 and 500 mg/kg) was administered intravenously. In other experiments suramin was given intracerebrally (100 to 250  $\mu$ g in multiple injections) to rats.
- 3) Glycosaminoglycan content, composition, and molecular weight distribution, lipids including sphingolipids and the activities of a number of lysosomal enzymes including 8 required for the degradation of the glycosaminoglycans and 8 required for the degradation of sphingolipids were determined.

Major Findings:

Previously we have shown an accumulation of partially degraded GAG in the tissue from patients with MPS. This accumulation varied in kind and in quantity from organ to organ and from type to type of MPS. Regarding brain involvement, we found a 4- to 6-fold increase in the GAG content of the neuronal perikarya from patients with MPS IH, II, and IIIA, a large increase in the gangliosides GM<sub>2</sub>, GM<sub>3</sub> and GD<sub>3</sub> and of ceramide lactoside. All these patients had mental retardation. In contrast, the GAG and sphingolipid content and composition of the neurons from a patient with MPS IS, who was an adult of normal intelligence, was normal. Our results suggested that the partially degraded heparan sulfate and perhaps the dermatan sulfate which accumulate in the brain of patients with MPS IH, II, IIIA and IIIB may inhibit catabolic enzymes of various sphingolipids. In turn the accumulation of sphingolipids in the neurons may account, at least in part, for the occurrence of neurological signs and progressive dementia in patients with MPS IH, II, IIIA and IIIB.



1. Tissues were examined from a patient with the tentative diagnosis by the pathologist of "a case of lipidosis." The results of chemical analysis suggested that we were dealing with an MPS. The liver was found deficient in  $\alpha$ -N-acetylglucosaminidase and the correct diagnosis was established as MPS IIIB (Sanfilippo B). The biochemical changes in the brain of this rare case of MPS IIIB were as follows: The total GAG and heparan sulfate contents were about 2.5 and 10 times greater, respectively, than that of the unaffected controls. Percentage distribution of the gangliosides showed that the N-acetylneuraminic acid of  $G_{M2}$  and  $G_{M3}$  comprised 13.4% and 13.2% of all the ganglioside compared with less than 1% in the control brain. Ceramide dihexoside was also increased. This patient was a 17-year old severely mentally retarded woman. These results are similar to those obtained previously from mentally retarded patients with MPS IH, II, IIIA and IIIB, and support our earlier conclusions.
2. The accumulation of GAG in the suramin-treated rat could be explained from the inhibition of the enzymes iduronate sulfatase,  $\beta$ -glucuronidase and hyaluronidase by the drug but the cause of accumulation of the gangliosides  $G_{M2}$  and  $G_{M3}$  was not known. We have now shown that 3 to 9 days after administration of suramin 500 mg/kg I.V. in rats, an amount of the drug about 0.25, 0.25, and 1.2  $\mu$ moles/g tissue was retained in the liver, spleen, and kidney respectively. Suramin, in vitro is a weak inhibitor of the sphingolipid hydrolases glucocerebrosidase, galactocerebrosidase,  $\alpha$ -galactosidase and arylsulfatase A (less than 50% inhibition at  $10^{-3}M$  concentration of the drug). The activities of  $\beta$ -galactosidase and sphingomyelinase were inhibited about 75% by  $10^{-4}M$  and  $5 \times 10^{-4}M$  suramin respectively. Suramin is a potent inhibitor of  $\beta$ -hexosaminidase (about 70% inhibition by  $10^{-5}M$  and 85% inhibition by  $10^{-4}M$  suramin). The activity of  $G_{M3}$ -sialidase was inhibited 80% by  $10^{-4}M$  suramin. The inhibition of  $\beta$ -hexosaminidase and  $G_{M3}$ -sialidase may explain the accumulation of  $G_{M2}$  and  $G_{M3}$  gangliosides in the brains of rats treated intracerebrally by suramin.

#### Significance to Bio-Medical Research and the Program of the Institute:

The mucopolysaccharidoses are inborn errors of metabolism resulting in lysosomal storage disease. The pathogenesis of these disorders is poorly understood. If inroads to therapy are to be made, a more complete knowledge of the mechanisms of disease will be required. To this end, these studies have contributed additional observations to the molecular biology and pathology of the mucopolysaccharidoses. As data of this sort accumulate, a better understanding of the pathogenesis will result.

The studies of the suramin model will assist in the understanding of the pathogenesis and describing secondary changes. Data indicates that accumulation of the drug and the attendant changes in MPS and sphingolipids may be reversible. If this is correct, reversibility of lesion maybe possible in this model and, perhaps, in the true disease state. These aspects may have profound implications for therapeutic endeavors in these disorders.

Proposed Course of the Project: We expect to extend our studies to the remaining types of MPS and to isolate lysosomes from tissues of the patients in order to study the biochemical changes in these primary loci of the disorders. We have evidence that in addition to ceramide lactoside other neutral hexosyl ceramides (possibly polyhexosyl ceramides) accumulate in the brains of the mentally retarded patients with MPS. We plan to study further the sphingolipids in MPS. Our ultimate goal is the study of the detailed chemical structure of the accumulated GAG fragments with the hope to correlate with specific dysfunctions, (e.g., organomegaly, corneal opacity, mental retardation, bone involvement, dysmorphism, etc.).

Regarding the suramin animal model, we propose to (i) study the time and dose response of the drug; (ii) to study the reversibility of the biochemical changes caused by suramin; (iii) preliminary observations show that suramin causes organomegaly in the rat. We intend to further investigate this organomegaly and its relationship to the organomegaly observed in storage disorders; (iiii) it appears that suramin inhibits differentially the isozymes of  $\beta$ -hexosaminidase,  $\beta$ -hexosaminidase A and  $\beta$ -hexosaminidase B. We plan to explore this observation and to evaluate its potential use in the study of lysosomal enzymes.

#### Publications:

1. Hadfield, M. G., Ghatak, N. R., Nakoneczna, I., Lippman, H. R., Myer, E. C., Constantopoulos, G. and Bradley, R. M.: Pathologic findings in mucopolysaccharidosis Type IIIB (Sanfilippo's Syndrome B). Arch. Neurol. 37: 645-650, 1980.
2. Constantopoulos, G., Rees, S., Cragg, B. G., Barranger, J. A. and Brady, R. O.: Effect of suramin on the activities of degradative enzymes of sphingolipids in rats. Res. Commun. Chem. Path. Pharmacol. 32: 87-97, 1981.

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SUMMARY OF WORK (200 words or less - underline keywords) <p>             The pathogenesis of Gaucher's disease is not understood. Previous work in this laboratory has identified the deficiency of <u>glucocerebrosidase</u> and the accumulation of glucocerebroside exclusively in <u>macrophages</u> of the reticuloendothelial system. The enzyme has been isolated and <u>purified</u>. Clinical trials of <u>enzyme replacement</u> have been encouraging and are expected to lead to a useful <u>therapeutic application</u>. Further study of the enzyme and the principles governing its incorporation into cells and activity towards stored glucocerebroside are required. A broader understanding of the disease and the <u>mechanisms</u> involved in <u>substrate storage</u> leading to <u>clinical symptoms</u> will be <u>necessary</u> to direct <u>therapeutic strategies</u>. The development of a <u>tissue culture model</u> of the disease using human macrophages will assist in these approaches to <u>elucidating important parameters</u> in the pathogenesis and treatment strategies.           </p>																																						

Project Description:

The project is divided into biochemical and clinical studies.

Biochemical Objectives: Further purify glucocerebrosidase and characterize properties of purified enzyme including multiple enzymatic forms, electrophoretic and isotachophoretic properties. We will also characterize enzyme purified from Gaucher patients, develop affinity column for enzyme isolation using antibodies and develop monoclonal antibodies to the enzyme. We expect to employ monoclonal antibody for: a) RIA or ELISA for diagnosis of enzyme deficiency; b) Tracing exogenous enzymatic activity through cells; c) Immunologic dissection of enzyme structure. We plan to use the antibody to assist collaborative effort to isolate functional genome (DNA) of glucocerebrosidase and to study processing of enzyme molecule. We will perform carbohydrate analysis and sequence of the enzyme and amino acid analysis and sequence of enzyme. We hope to be able to determine what structural features of the enzyme are important in terms of: a) stability; b) efficient incorporation by cells, particularly the macrophage; c) tissue survival time; d) interaction with stored glucocerebrosidase. Considerable effort will be directed towards developing a tissue culture model using Gaucher macrophages. We expect to employ this culture model to study consequences of the structural features of the enzyme and its disposition by cells. The culture model may also be helpful in elucidating the importance of the macrophage in the pathogenesis of the disorder. We will appraise the effects of glucocerebrosidase storage on several functions of the macrophage. In this regard we will employ the culture model to evaluate the importance of the macrophage and storage material in bone resorption and formation.

Clinical Objectives: In order to appraise the pathogenetic mechanisms at work in this disease, an array of clinical studies will be required to fully evaluate the organ systems involved. An attempt will be made to introduce the macrophage as the culprit in the protean manifestations of the disorder. Thus, the unifying hypothesis is that Gaucher's disease is a macrophage-mediated disorder. The consequences of this hypothesis, should it be supportable, are significant in terms of directing enzyme replacement therapy towards these cells.

Major Findings: Glucocerebrosidase has been purified to a specific activity of  $7 \times 10^6$  U/mg protein. This is seven fold better than our previous preparation and forty times better than any published preparation by other investigators. The enzyme has a molecular weight of 60,000-70,000 daltons. Various conditions, especially alkaline pH, cause the molecule to aggregate and lose activity. At least four forms of the enzyme are discernable by isoelectric focusing. One of these forms, the most acidic, appears to be relatively more important in Gaucher's disease. Antibodies have been developed against the enzyme. These are being used to isolate DNA and to study the processing of the enzyme. Glycosidase treatment of the enzyme indicates the presence of sialic acid, galactose, N-acetylglucosamine, and mannose in the molecule. These sugars have been demonstrated to alter the clearance and distribution of the enzyme. Glycosidase treatment results in more efficient delivery of enzymatic activity to the macrophage and target cell in Gaucher's disease indicating naturally occurring lectins can be utilized to effect more



efficient uptake of exogenous enzyme. Some antiglycocerebrosidase activity has been seen in mouse myeloma cultures. Further work is needed to better identify and harvest this activity so that animals with high titers of antibody in peritoneal fluid can be produced. Gaucher monocytes mature into macrophages in several days. Their enzymatic assay differs somewhat from the assay in other tissue.

#### Significance to Bio-Medical Research and the Program of the Institute:

Gaucher's disease is the prototype disorder for the application of enzyme replacement techniques. Several animal and tissue culture models including the mucopolysaccharidoses have given significant impetus to the idea being reasonable and practical. The initial clinical studies in Gaucher's disease provided the first evidence that tissue storage in a human disease could be reduced by infusion of enzyme. Trials have been extended to multiple infusions on a chronic basis. The results of these studies are pending. Simultaneously, animal studies have revealed that only small amounts of infused enzyme actually reaches the target cells. The delivery can be increased about five fold by appropriate modification of the enzyme. Moreover, the disease is being more broadly studied so that a better understanding of its manifestations and possible alterations by the therapy can be made.

Should the hypothesis be established that enzyme replacement can reverse or alter the course of Gaucher's disease, the possibility of applying this technique to other lysosomal storage disorders could be examined. This would provide a useful treatment of an entire group of untreatable and disabling or fatal diseases.

Proposed Course of the Project: The project will focus on a broad understanding of the abnormal biochemistry of Gaucher's disease particularly in reference to what governs the delivery of exogenous enzyme to cells and the interaction with its substrate.

#### Publications:

1. Ginns, E. I., Brady, R. O., Stowens, D. W., Furbish, F. S. and Barranger, J. A.: A new group of glucocerebrosidase isozymes found in human white blood cells. Biochem. Biophys. Res. Commun. 97: 1103-1107, 1980.
2. Furbish, F. S., Steer, C. J., Krett, N. L. and Barranger, J. A.: Uptake and distribution of placental glucocerebrosidase in rat hepatic cells and effects of sequential deglycosylation. Biochim. Biophys. Acta 673: 425-434, 1981.
3. James, S. P., Stromeyer, F. W., Chang, C. S. C. and Barranger, J. A.: Liver abnormalities in Gaucher's disease.. Gastroenterology 80: 126-133, 1981.









# ANNUAL REPORT

October 1, 1980 through September 30, 1981

Laboratory of Neuropathology and Neuroanatomical Sciences  
National Institute of Neurological and Communicative Disorders and Stroke

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Laboratory of Neuropathology and Neuroanatomical Sciences, IRP  
National Institute of Neurological and Communicative  
Disorders and Stroke

Igor Klatzo, Chief

For the past year, all Sections of the LNNS showed a considerable progress in widening the scope of conducted investigations. This was achieved by tackling new areas of research and by applying new methodological approaches.

The Section on Cerebrovascular Pathology has considerably increased its research horizons by acquiring the potential for quantitative assessments of regional cerebral blood flow (rCBF). The one approach, based on collaboration and assistance from the Laboratory of Cerebral Metabolism, NIMH, permits carrying out computerized quantitative radioautographic assays on the rCBF, according to the Sokoloff method. The other approach, which recently became available, is the estimation of rCBF by hydrogen clearance using implanted platinum electrodes.

Independently, the Section has developed a standardized specific gravity (SG) method for a sensitive evaluation of changes in water content in the brain tissue. This method is very essential for recognition of early and/or slight changes in water content and, using tissue samples only 1 mm in diameter, it is possible to map out regionally the dynamics of the edematous process. The factors which were discovered to affect the reproducibility of the SG method were the impurities in the kerosene, the size of the samples, and the temperature. Strict control of these factors and standardization make this method most reliable and valuable in our studies on brain edema.

Combining the rCBF determinations, estimation of water content by SG and evaluation of the blood-brain barrier (BBB) changes by Evans Blue (EB) tracer and by the immunocytochemical procedure of peroxidase-antiperoxidase (PAP), it was possible to determine the regional and chronological interrelationships between these changes in cerebral edema produced by occlusion of the middle cerebral artery (MCA) in cats. Our observations indicated that initially the ischemic edema is of purely cytotoxic character and it develops in areas with rCBF below a certain threshold. Changes in the BBB, allowing escape of serum proteins, introduce the vasogenic component of ischemic edema which is not dependent on any rCBF threshold. The observations revealed that the extravasated serum proteins in the gray matter tend to shift into the adjacent white matter aggravating and changing the dynamics of ischemic edema. The observations on vasogenic brain edema due to cold lesion in cats revealed that spreading of edema through the white matter may produce, presumably by compression of microcirculation, a significant reduction in rCBF in the adjacent and even relatively distant areas of the gray matter. A finding, of potential clinical significance, in studies on ischemia due to occlusion of the MCA in cats was that even after a complete healing of the ischemic lesion there may remain a slight but significant reduction of CBF in the ipsilateral hemisphere as long as one month after MCA occlusion.

Comparative, concurrent evaluations of rCBF, SG, and BBB changes in the same tissue were extended into the field of epileptic seizures. Pharmacologically-induced epileptic activity may be centered in various regions of the brain, depending on the particular agent used. The rCBF assays on 18 different regions of the brain revealed that the degree of rCBF increase and its regional localization at the onset of epileptiform seizures depend on the type of seizure induction and on the level and rate of concurrent elevation of the systemic blood pressure (BP). Thus, an abrupt blockage of GABA-ergic transmission by bicuculline induces very high increases of rCBF in pallidum and thalamus and also in preoptic area and brain stem regions, whereas a gradual cessation of GABA-ergic transmission by methoxypyridoxine affects mainly - besides pallidum and thalamus - caudate nucleus and hippocampus, but virtually omits brain stem areas. In contrast, enhancement of glutamergic transmission by kainic acid elevates the rCBF to a lesser extent and exclusively in forebrain regions. The regional correlation of the rCBF changes occurring in drug-induced epileptiform seizures and in acute hypertension with the regional changes in permeability of the BBB revealed the following. In acute hypertension, the EB leakage was confined to occipital cortex and superior colliculus and it could be produced only in rapidly induced hypertension by adrenalin. In drug-induced epileptiform seizures, the breakdown of the BBB did not require such high BP elevations and was correlated with regional CBF elevations observed with the respective agents. An unexpected finding was the observation that rCBF elevations and BBB changes are not associated with local lowering of SG, as should be expected with an onset of edematous changes, but on the contrary an increase in SG was regularly found, suggesting, perhaps, an increase in osmolarity in these regions.

For the past year, the Section on Neurocytobiology has continued to investigate: I. The altered blood-brain barrier (BBB) and metabolic state in cerebral ischemia. II. The biochemical, histochemical and morphological properties related to the transport and/or metabolism of cerebral capillaries, pia-arachnoid and neurons in vivo and in vitro which include studies in tissue culture.

I. 1) The continuous effort in evaluating the beneficial influence of the naturally occurring CNS depressants [ $\gamma$ -butyrolactone (GBL) and  $\gamma$ -hydroxybutyrate (GHB)] on cerebral ischemia has been focused on elucidating further the mechanism responsible for the GHB action on cerebral ischemia in regard to the synthesis of neurotransmitters by investigating the activity of tyrosine and tryptophan hydroxylase. These studies revealed that the cerebral ischemic reduction in the activity of tyrosine and tryptophan hydroxylase can be prevented by the administration of GHB prior to the ischemic insult. Hence, it might also prevent the decrease of monoamines observed in cerebral ischemia and subsequent secondary injury to the brain.

2) The pathogenetic investigations of cerebral ischemia and ischemic edema have been concerned with determining the effect of this disease process on the neurotransmitter receptors, in particular the  $\beta$ -adrenergic type. These studies included the analysis of  $\beta$ -receptors' properties in the normal gerbil's brain since this information was unavailable until now.

a) Normal brain: The membranous fraction ( $P_2$ ) obtained from the cortex and hippocampus showed a specific (saturable) binding site for  $^3\text{H}$  dihydroalprenolol ( $^3\text{H}$ -DHA) with an apparent dissociation constant ( $K_D$ ) 1.0 mM and an apparent binding maximum ( $B_{\text{Max}}$ ) 136/fmol/mg protein. The Hill plot analysis revealed a single binding site. The analysis of the subtypes of  $\beta$ -receptors had shown the presence of both  $\beta_1$  and  $\beta_2$  adrenergic receptors with a predominance of  $\beta_2$  receptors.

b) Cerebral ischemia led to a change in the membrane affinity for  $^3\text{H}$ -DHA binding sites due to a decreased association but not dissociation rate of the ligand to the  $\beta$ -receptors. At the same time the affinity of the receptors for norepinephrine was also altered slightly in the ischemic membrane. Moreover, the effect of guanyl nucleotides on the agonist (isoproterenol) binding was found to be decreased in ischemic membrane. The findings suggest that ischemia disturbs the regulatory mechanism of the  $\beta$ -receptors.

II. 1) Further studies related to the elucidation of the mechanism responsible for the limited BBB passage of monoamines and their metabolites have shown that the isolated microvessels which take up norepinephrine (NE) and 5-hydroxytryptamine (5-HT) by specific  $\text{Na}^+$ - and  $\text{K}^+$ -dependent carrier mediated process are unable to handle their metabolites the same way. The normetanephrine, metanephrine and 5-HIAA displayed a diffusible character, since their capillary uptake could not be influenced either by self inhibition or cross inhibition by other amines or by hypothermia. However, the uptake and/or the metabolism of normetanephrine and metanephrine was enhanced by the same bivalent ions ( $\text{Co}^{++}$ ,  $\text{Mn}^{++}$ ,  $\text{Mg}^{++}$ ,  $\text{Cd}^{++}$ ) as those of NE. On the other hand, these cations had little if any effect on 5-HT uptake but they markedly reduced the 5-HT metabolism. These findings together with the observed time-dependent accumulation of the amines' metabolites in the incubation medium and not in the capillaries indicate that the cerebral microvessels are incapable of retaining the metabolic product of NE and 5HT.

2) a) The continuous studies concerning the properties of endothelial cell cultures derived from dissociated cells of cerebral microvessels have demonstrated a specific endothelial plasma membrane antigen in these cells by immunofluorescence. The antigenic reactivity was manifested by a specific diffuse surface fluorescence of the endothelial cells when treated with rabbit antiserum prepared against endothelial membrane fraction. A strong mitochondrial fluorescence was also observed but it was shown to be, in part, nonspecific by normal serum and phosphate buffered controls. The indirect immunofluorescence investigations were also applied to frozen sections of the brain showing specific reactivity of endothelial cells only. Thus, this antigenic property of the endothelial cells provides a useful and reliable marker for the further study of capillary endothelium in the living state under normal and pathological conditions.

b) Attempts have been made to elucidate the functional interrelationship of nervous tissue and vascular endothelium since ontogenetically the cerebral microvessels acquire their specific BBB-related function only after they penetrate the brain from the periphery. These studies so far have demonstrated



a stimulation of endothelial cell (derived from the established endothelial cell cultures of cerebral microvessels) growth co-cultured with explants of cerebellum as judged by cellular incorporation of  $^3\text{H}$  thymidine radioautographically. Moreover, the activity of the alkaline phosphatase (which has been known as a vascular marker) was markedly increased in the endothelial cells co-cultivated with organotypic cerebellar cultures. This system will be used for an extensive exploration of the effects of nervous tissue on the enzymatic and morphologic properties of capillary endothelium.

In the Section on Cellular Neuropathology, investigators use immunocytochemical techniques to study the distribution of myelin and glial constituents in experimental and human demyelinating diseases.

Two projects compare the distribution of proteins in compact myelin [basic protein (BP) in the central nervous system (CNS);  $\text{P}_0$ , BP and  $\text{P}_2$  in peripheral nervous system (PNS)] and of myelin-associated glycoprotein (MAG), a constituent of myelin forming glial cells (oligodendroglia in CNS; Schwann cells in PNS). In the first project, these distributions were compared in optic nerve demyelination secondary to axonal transection (Wallerian degeneration). Electron microscopic changes were identified in some axons 24 hrs. after eye removal and they preceded light microscopic alterations in the distribution of MAG and BP, MAG, and BP alterations appeared simultaneously 48 hrs. after transection when electron microscopic changes in axonal organelles and axolemma were more advanced. Subsequently, they progressed in parallel with the secondary degeneration of myelin sheaths.

Segmental myelin breakdown with relative sparing of axons occurs in idiopathic polyneuritis, a debilitating human neuropathy characterized by inflammatory cell infiltrates and demyelination. In early lesions, there were focal alterations in the distribution of three compact myelin proteins -  $\text{P}_0$ , BP, and  $\text{P}_2$ . Where these abnormalities were identified, there also were changes in MAG distribution. More severe, later changes in the distribution of  $\text{P}_0$ ,  $\text{P}_2$ , BP, and MAG were consistent with the pattern of myelinated fiber changes seen in segmental demyelination and Wallerian degeneration. In regenerating fibers, MAG antiserum stained periaxonal regions intensely; thin regenerating myelin sheaths were stained by  $\text{P}_0$  and BP but not by  $\text{P}_2$  antiserum.

Another important project concerned the localization of  $\text{P}_0$  in Schwann cells during myelin formation. To localize  $\text{P}_0$ , its distribution in immunostained  $1\ \mu\text{m}$  sections was mapped on electron micrographs of identical areas found in adjacent thin sections. The first  $\text{P}_0$  staining was detected around axons and/or in cytoplasm of Schwann cells that had established a 1:1 relationship with axons. As myelination progressed, intensely stained myelin sheaths were more numerous and in adult nerves, all sheaths were densely and uniformly stained. Particular  $\text{P}_0$  staining was observed also in juxtanuclear areas of Schwann cell cytoplasm. It was most prominent during development, then decreased, but still was detected in adult nerves. The cytoplasmic areas stained by  $\text{P}_0$  antiserum were rich in Golgi membranes.

These observations demonstrate that immunocytochemical methods are useful in exploring cellular mechanisms of myelin formation, maintenance and breakdown in human and experimental demyelinating diseases.

The main goal of the Section on Functional Neuroanatomy is to understand synaptic transmission and other aspects of neuronal function by means of new structural techniques. In the course of studying release of transmitter at synapses, an important new technique for freezing tissue directly was developed (as an extension of Van Harrevelde's early work). These studies of transmitter release are completed and our current program depends on exploring several new avenues opened by the freezing technique.

The first advantage of the direct freezing technique, which led to its development, is that rapid structural changes can be stopped with a msec time resolution. In the last year, the Section has published major papers showing the fate of synaptic vesicle membrane following exocytotic transmitter release, and how exocytosis begins as a punctate rearrangement of the plasma membrane in a cell from Limulus especially suited to this purpose.

A second advantage of the freezing technique is that soluble components of the cell interior are preserved in their natural positions. Development has continued on a promising approach to measure the amount and distribution of intracellular calcium in tissues with an electron microprobe. The remaining problem, which seems to be close to solution, is diffusion of that cytoplasmic calcium which is not confined in membrane-bound organelles. This artifact is greatly reduced by combining new cryoembedding techniques (developed in Kellenberger's laboratory) with very low temperature freeze-substitution in a special solvent (tetrahydrofuran) developed in the Section on Functional Neuroanatomy. This approach has proven to be useful also for locating deoxyglucose, and its application to soluble cytoplasmic proteins and peptides is being tested.

Direct freezing can also be used to visualize intramembrane proteins in greater detail and closer to their natural state, in order to understand how they function as channels. For this purpose a special apparatus has been developed to freeze-fracture tissue at temperatures near absolute zero (10°K). This approach prevents many of the structural changes which normally occur during fracturing and shadowing. Application of this technique to open and closed channels ("connexions") at gap junctions shows new structural details which change depending on functional state.

The last application for freezing that is being explored in the Section is to study insoluble components of the cytoplasm, the "cytoskeleton". The new freeze-fracture technique allows the inside of axons to be visualized without any of the chemical pretreatments that have been used up to now to prepare cytoskeletons. This approach shows that organelles involved in axoplasmic transport are situated in special "compartments" of the axoplasm and that each type of organelle has characteristic relationships with cytoskeletal elements. This approach has been applied in collaboration with Dr. Robert Gulley to show the relationships of the cytoskeleton to the postsynaptic membrane of auditory brain stem synapses. Fine filaments connect components of the postsynaptic membrane, believed to be receptors, with an actin network lying in the cytoplasm beneath the synapse. This finding explains the long-term stability of the postsynaptic region of the neuronal membrane.

Other work in the Section, which does not depend on the freezing technique, also serves as training projects for Fellows. The membrane ultrastructure of synapses on tonic (slow) muscle fibers has been seen for the first time. A major paper is in press on the changes in synaptic membrane structure (as seen with freeze-fracture) that occur during nerve degeneration. Our scanning electron-microscope facility has been used to define the response of muscle satellite cells to muscle injury, and to develop a new way to look at the interior of the brain to see more clearly details of cellular relationships there.

The Section on Neurocytology has extended its investigations of regeneration of peripheral and central neurons to include changes in a neuronal enzyme, the manipulation of particle assemblies within astrocyte membranes and the by-passing of the blood-cerebrospinal fluid (CSF) barrier via ganglion transplants. It was found that sensory ganglia, such as dorsal root ganglion (DRG), like autonomic ganglia, such as superior cervical ganglia (SCG), when transplanted to the undamaged ventricular surface are engulfed by choroid plexus. This peculiar response of an intact epithelium held together by tight junctions is thus triggered by sensory as well as autonomic ganglia. Like the SCG, the DRG can survive for months in the cerebrospinal fluid compartment.

It is now asked whether there is specificity between axon terminals and the blood vessel walls upon which they terminate. Allografts of fetal hypothalamus are placed over the area postrema (AP) in rats. The normal target of hypothalamic neurosecretory (NS) neurons is fenestrated blood vessels of the neurohypophysis and median eminence. Such vessels are in the AP, which is not a normal target. Can central nervous system (CNS) neurons regenerate onto AP or other fenestrated vessels to produce functional neurohemal contacts? Three months after transplantation, the hypothalamic grafts remain large and crowded with neurons and a rich neuropil including numerous axo-somatic synapses. Some axons contain what appear to be large neurosecretory droplets in addition to synaptic and dense-core vesicles.

The blood-CSF barrier to horseradish peroxidase (HRP) can be circumvented by transplanting neural tissue with fenestrated vessels to the brain surface. Such vessels within the SCG are permeable and, as they do in situ, allow HRP to escape into the interstitial spaces of the ganglion. These spaces become confluent with those of the cerebral parenchyma and, within 10 minutes, HRP moves from blood to brain via the spaces. Thus, without injury to the brain surface, blood-borne macromolecules can be introduced into the extracellular fluid of the brain.

The glycolytic enzyme, neuron-specific enolase (NSE) has been followed immunocytochemically during nerve degeneration and regeneration. Is there a switch from NSE to non-neuronal enolase (NNE) during degeneration as was found during development? It is now noted that the levels of NSE in the hypoglossal nucleus of the rat fall during the first 10 days after nerve crush and return to normal levels some 35 days later. The next steps are to see whether there is a concomitant rise and fall in NNE which would intimate a "de-differentiation" of the neuron during degeneration, and to test the hypothesis that recovery of normal NSE levels depends on re-occupation of vacated synaptic sites.

The association of the orthogonal assemblies of particles within the plasma membrane of astrocytes and the cytoplasmic matrix has been borne out by several lines of evidence. The particles are invariably associated with the P or cytoplasmic face of the membrane but never the external, E face. The distinctive changes in the distribution of assemblies within astrocyte cultures have been obtained with a number of agents that affect the cytoskeleton. After exposure to cytochalasin, which affects actin filaments, the assemblies clump so as to form large rafts throughout the membrane. With colchicine and vinblastine, which disaggregate tubulin, much of the membrane is free of assemblies that have migrated to one region of the membrane to form a "cap" over the cell. When a localized cold lesion of the brain is made in young rats, the assemblies at the lesion site are indistinguishable from those that have been denatured by urea or guanidine: the assemblies become so tightly packed that all background particles are excluded. In astrocytes at the periphery of the lesion, the assemblies increase in number but do not clump. The rearrangement of assemblies by the above agents strongly intimates a linkage to cytoplasmic proteins and shows that the effect of a cold lesion is comparable to that of denaturation.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02425-02 LNNS																
PERIOD COVERED October 1, 1980 to September 30, 1981																		
TITLE OF PROJECT (80 characters or less) Glial fibrillary acidic protein (GFAP) reaction in astrocytes																		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																		
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SECTION Section on Cerebrovascular Pathology																		
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CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS         </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div> <div style="display: flex; justify-content: space-between; align-items: flex-start; margin-top: 5px;"> <div> <input type="checkbox"/> (a1) MINORS         </div> <div> <input type="checkbox"/> (a2) INTERVIEWS         </div> </div>																		
SUMMARY OF WORK (200 words or less - underline keywords) This project has been terminated.																		



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PERIOD COVERED October 1, 1980 to September 30, 1981																		
TITLE OF PROJECT (80 characters or less)  Studies on cerebral embolism produced by injection of microspheres into the internal carotid artery in cats																		
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PERIOD COVERED October 1, 1980 to September 30, 1981																										
TITLE OF PROJECT (80 characters or less) The regional selectivity of blood-brain barrier (BBB) changes induced by various epileptogenic agents and acute hypertension																										
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SUMMARY OF WORK (200 words or less - underline keywords) The changes of the BBB to Evans Blue (EB) and horseradish peroxidase (HRP) were studied in <u>epileptic seizures</u> induced by different agents and in <u>acute hypertension</u> . The findings, indicating a great regional variability in distribution of the BBB lesions in relation to a type of epileptogenic agent, suggest that local neurotransmitter contents and receptor sensitivity may be instrumental in BBB changes in addition to purely vascular effect of hypertension.																										

## Project Description:

**Objectives:** The breakdown of the blood-brain barrier (BBB) to proteins has been described both in epileptic seizures and in acute hypertension. In order to elucidate further the pathophysiological mechanisms responsible for protein extravasation, barrier changes to Evans Blue (EB) and horseradish peroxidase (HRP) tracers were studied with regard to their incidence and localization following induction of the seizures by different agents and in acute hypertension produced by injection of adrenaline.

**Methods Employed:** Thirty-six New Zealand white male rabbits were used. In animals destined for blood pressure (BP) measurements, the right femoral artery was cannulated 1 to 2 days before the experiment under ether anesthesia. All injections to induce seizures or hypertension were done via the ear vein in fully conscious animals kept in a rabbit restrainer. As tracers for the opening of the BBB, either EB (visualized macroscopically and fluorescence-microscopically) or HRP (visualized after histochemical staining both light- and electron-microscopically) were given at various time intervals before and after seizure induction or hypertension. At the end of the experiment, the rabbits were perfused trans-aortically under Nembutal anesthesia with aldehyde fixatives.

The following drugs were used: (1) Adrenaline-chloride, either alone or in combination with atropine in a dosage sufficient to induce an abrupt rise of at least 100 mm Hg in the mean arterial BP. (2) Pentylenetetrazole, a potent convulsant drug which induces seizures resembling human grand mal epilepsy. (3) Bicuculline, which acts as a GABA-receptor blocker. (4) Methoxypyridoxine, an inhibitor of GABA synthesis. (5) Kainic acid, a glutamate-receptor mimeticum. (6) L-methionine-D,L-sulfoximine, an inhibitor of glutamate degradation.

**Major Findings:** (a) In acute hypertension experiments, the breakdown of the BBB could only be observed when the mean arterial BP rose for at least 100 mm Hg in 10 sec after the bolus injection, and provided that bradycardia was prevented with the help of atropine. The BBB breakdown to EB was localized exclusively in neocortical areas, mostly as small blue spots in the parietal and occipital cortices. The EB spots were rarely bilateral and symmetrical. The opening of the BBB lasted only during the peak of hypertension, i.e. about 1 minute.

(b) Pentylenetetrazole-induced seizures in combination with an increase in BP of 30-50 mm Hg, as well as severe convulsions induced by the other drugs, caused a bilateral BBB breakdown along the whole brain basis: piriform cortex, septal areas, preoptic areas, pallidum, hypothalamus (sparing the corpus mammillare), thalamus (sparing the center median), periaqueductal gray and surrounding reticular formation, inferior colliculus, basis of the cerebellum, and rhombencephalon. Leakage in parietal and occipital cortices, as well as caudate nucleus and superior colliculus, was never observed. The opening of the BBB lasted only for 3 to 5 minutes after seizure onset.

(c) Mild bicuculline-seizures resulted in a selective and strongly symmetrical breakdown of the BBB in globus pallidus. After more severe seizures, a similar pattern of leakage as during pentylenetetrazole-seizures was seen, including staining of the hippocampus.

(d) Methoxyypyridoxine-seizures produced the EB leakage preferentially in the hippocampus.

(e) Lethal kainic acid seizures resulted in spotlike nonsymmetric EB leakages in cortical and thalamic areas.

(f) Methionine-sulfoximine seizures induced a very selective BBB breakdown restricted to the corpora mammillaria, an area resistant to all other forms of treatment. Only after lethal seizures was it possible to see additional EB leakages in hypothalamus and brain stem areas.

Electron microscopical investigations revealed that the macromolecular HRP was transported into the neuropil by an increased pinocytosis in the endothelial cells. This event was preferentially taking place at the level of the arterioles. There was no indication of opening of the tight junctions.

Significance to Biomedical Research and the Program of the Institute:

The presented findings concerning the regional selectivity of the BBB changes to proteins indicate that, although a certain elevation in the mean arterial BP is a prerequisite for the barrier breakdown in epileptiform seizures, the high BP does not determine the locus of BBB damage. The extravasations of EB in acute hypertension without seizures were associated with relatively high elevations of BP and were of different distribution than those observed in seizures where the localization of BBB lesions seemed to depend in a large degree on the mode of seizure induction and on the neurotransmitter system involved. Thus, the drugs affecting the GABA-system resulted in primary lesion in pallidum and hippocampus, whereas compounds affecting the glutamate transmission involved primarily the thalamic areas. These findings imply that not only vascular factors determine BBB changes but that possible regional changes in extracellular neurotransmitter content or receptor sensitivity may determine the patterns of BBB breakdown.

Proposed Course of the Project: The findings of this project provide a basis for the correlation of regional BBB changes with regional cerebral blood flow changes in epileptic seizures.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02457-01 LNNS																
PERIOD COVERED October 1, 1980 to September 30, 1981																		
TITLE OF PROJECT (80 characters or less) Regional cerebral blood flow (rCBF) changes in variously induced epileptiform seizures																		
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SUMMARY OF WORK (200 words or less - underline keywords)  The local changes in CBF were assessed in various regions of the brain of rabbits subjected to <u>epileptic seizures</u> induced by various agents. There were distinct patterns of rCBF changes with regard to each agent used. The correlation of rCBF changes with regional alterations of the BBB in various types of seizures should further elucidate the underlying mechanisms involved.																		



## Project Description:

Objectives: Induction of epileptic seizures by various agents is associated with marked regional changes in neuronal metabolism and in regional cerebral blood flow (rCBF). Since the rCBF changes may play an important role in abnormal BBB permeability, the rCBF was studied in 18 different brain regions of the rabbits at the onset of generalized seizures induced by several convulsant drugs working via different action mechanisms. It was intended to use these detailed data on rCBF as a base for comparative studies with regional BBB changes.

Methods Employed: In 29 male New Zealand white rabbits, 2.0 to 2.6 kg in weight, the left femoral artery was cannulated for continuous blood pressure (BP) recording, the right femoral vein for infusions, and the left brachial artery for blood sampling. Electroencephalogram (EEG)-electrodes were fixed over the fronto-parietal skull and the EEG was recorded continuously. When all surgical procedures were completed, the anesthesia was terminated. After immobilization with Flaxedil, the rabbits were artificially respirationed.

When the EEG-recordings indicated full recovery from the effects of anesthesia (this was usually the case after 80 to 120 minutes), the head of the rabbit was mounted in a guillotine. Via the ear vein, 2 ml of saline or 2 ml of a convulsant drug (0.2 mg/ml bicuculline, 80 mg/ml methoxyypyridoxine, 50 mg/ml kainic acid) was rapidly injected. After 10 seconds, or when the EEG recording indicated seizure onset, 280  $\mu$ ci of  $^3$ H-nicotine bitartrate dissolved in 6 ml of saline were infused via the femoral vein at a constant rate over 40 seconds. During the infusion, arterial, and whenever possible, jugular venous blood samples were collected every 2 to 4 seconds. Exactly 40 seconds after the start of the tracer infusion, the rabbit was decapitated.

Thirty regional brain areas were dissected out, weighed (range 10 to 40 mg), and dissolved in 2 ml Soluene 350 overnight. Twenty  $\mu$ l aliquots of the blood samples were treated identically. After addition of 10 ml Aquasol, the radioactivity of the blood and brain samples was counted in a liquid scintillation counter (Beckman). Applying the Kety-equations, the regional CBF could be estimated by interpolation from the blood curve.

Major Findings: The regional CBF estimated with the  $^3$ H-nicotine method in control rabbits (n=6) was in good agreement with data in rat and cat obtained with the  $^{14}$ C-iodoantipyrine method. Blood flow is highest in the cortical areas, the colliculi and caudate nucleus, and it is low in brain stem areas, the septal part of the hippocampus and the pallidum. The lowest value from all regions was found in the internal capsule.

During generalized seizures, CBF was considerably increased. The degree of increase and its regional distribution, however, depended on two factors: (1) presence or absence of an elevation of systemic arterial BP of at least 40 mm Hg, and (2) the type of convulsant used.

The highest increase in CBF occurred with the onset of bicuculline-seizures, if these were accompanied by a rise in mean arterial BP (n=5). The absolutely highest levels of CBF were found in frontal cortex, thalamus and inferior colliculus. The relative increase, on the other hand, was largest in pallidum with 534%, followed by the preoptic area (512%) and the periaqueductal gray (481%). The lowest percentage increase was found in the basal vermis of the cerebellar cortex (288%). When an increase in systemic BP was absent (n=1), CBF increased only 1 to 2 times, with the highest rates in thalamus and pallidum.

Methoxyypyridoxine-seizures without a BP rise (n=4) showed a comparable result: 1.2- to 3-fold increases in CBF, the highest in thalamus and pallidum. Methoxyypyridoxine-seizures associated with an elevation in systemic BP (n=6) induced a somewhat less conspicuous increase in CBF than did full-blown bicuculline-seizures. The highest relative rise in CBF was observed in pallidum (422%), followed by the thalamus (321%) and caudate nucleus (314%). Relatively high increases in CBF were also present in the hippocampal areas. The lowest rise with only 129% was found in the inferior colliculi.

Kainic acid-seizures (n=7) were preceded by a gradual increase in systemic BP to about 200/150 mm Hg. The seizure onset did not seem to induce a further rise in BP. Increases in CBF ranged between 350% (preoptic area, septum, hippocampal areas) and 100% (inferior colliculi, periaqueductal gray).

#### Significance to Biomedical Research and the Program of the Institute:

The determinations of rCBF revealed a different pattern of changes related to different epileptogenic mechanisms involved. The concurrent elevation of the systemic BP was associated with significantly increased rCBF values. These data provide a basis for correlative studies with regional BBB changes due to similarly induced forms of epileptic seizures.

Proposed Course of the Project: The data from the project will be used in a correlative study between rCBF changes and local alterations of the BBB in various types of epileptiform seizures.

Publications: None

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PERIOD COVERED October 1, 1980 to September 30, 1981																						
TITLE OF PROJECT (80 characters or less) Changes in specific gravity (SG) of rabbit brain tissue during drug-induced epileptiform convulsions																						
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SUMMARY OF WORK (200 words or less - underline keywords) <u>Changes in specific gravity (SG) reflecting the water content of the tissue were evaluated during drug-induced epileptiform convulsions and in acute hypertension. Contrary to the expectation of finding lower SG, i.e. increased water content in brain regions showing the blood-brain barrier (BBB) changes due to epileptic seizures or to acute hypertension, these areas revealed an increased SG, i.e. a relatively reduced water content.</u>																						

## Project Description:

Objectives: A blood-brain barrier (BBB) breakdown to proteins is associated in the majority of cases, as e.g. ischemia or cold lesion, by a regional vasogenic brain edema. The aim of the present study was to determine the degree of edema in brain areas exhibiting BBB leakage due to epileptiform convulsions. The most sensitive method to measure changes in brain water content is by estimating the specific gravity (SG) of small brain samples, and this method was applied in this study in rabbits subjected to various forms of epileptiform seizures and to acute hypertension. Additional experiments were carried out to obtain SG measurements in brains perfused with saline in order to estimate the participation of possible regional hyperemia, whereas the direct estimation of brain water content was calculated by wet/dry weight ratios.

Methods Employed: Thirty-six male New Zealand white rabbits, 2.0 to 2.7 kg, were used. For blood-pressure measurements, the femoral artery was cannulated 1 to 2 days previously. For the experiment, the rabbits were restrained on a table and received a single injection of 2 ml/kg 2% Evans Blue in order to verify the presence of BBB leakage. At the end of the experiment, the animals were decapitated or rapidly perfused with 200 ml saline under Nembutal anesthesia. The brains were dissected out and cut in coronal slices which were immersed in kerosene. The whole dissection procedure lasted between 2 and 2.5 min. Using a gradient column, SG was determined bilaterally in 15 different brain regions from control animals (treated exactly like experimental animals) and 3 and 20 min after 0.4 mg/kg bicuculline or 20 min after 50 mg/kg pentylenetetrazole. Possible changes due to hypertension were also checked in rabbits made hypertensive with adrenaline.

In addition, water content was determined in 4 brain regions of control rabbits and in animals subjected to 20 min bicuculline- or pentylenetetrazole-seizures. The wet tissue sample was weighed directly after dissection and then after drying in an oven set to 90°C until a constant weight was obtained (28 days).

Major Findings: No significant changes in brain tissue SG were observed after 3 min of bicuculline-seizures. When the convulsions lasted for 20 min, however, significant increases in SG were observed in all brain areas, the highest elevations being present in caudate nucleus, septum and cerebellar cortex, the lowest in internal capsule and rhombencephalon. After 20 min of pentylenetetrazole-seizures, the increases in SG values were even higher, especially in motor cortex, caudate nucleus, thalamus and cerebellum. In contrast, hypertension induced only minor increases in SG in septum and preoptic area.

Saline perfusion in control rabbits reduced the SG only in a few brain areas, i.e. cortex and cerebellum. Saline perfusion after 20 min of pentylenetetrazole-induced convulsions resulted in somewhat less pronounced elevations in SG when compared with normal controls. However, the increases were still significantly higher than the SG values of saline-perfused controls. This result implied that the increases in SG are not influenced by local hyperemia, or only to a very minor extent.

The direct estimation of water content with the wet/dry method in cerebellum, preoptic area, hypothalamus and hippocampus revealed a reduced water content in rabbit brain after 20 min of bicuculline- or pentylenetetrazole-seizures. However, due to the greater variability of this method, the reduction in water content was only significant in cerebellum after pentylenetetrazole-seizures.

Significance to Biomedical Research and the Program of the Institute:  
The evaluation of regional changes in water content by sensitive gravimetric method revealed an unexpected finding of an increased specific gravity in brain regions showing BBB changes due to epileptiform seizures. The direct water determinations supported this finding, suggesting some form of dehydration in the brain tissue affected by seizure activity. On the other hand, this could also be due to an accumulation of some substances, and to elucidate this finding, corresponding regional measurements of brain osmolarity are contemplated.

Proposed Course of the Project: The extension of this project to include regional changes in osmolarity will depend on development of appropriate methodology.

Publications: None



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02459-01 LNNS																								
PERIOD COVERED October 1, 1980 to September 30, 1981																										
TITLE OF PROJECT (80 characters or less) The correlation of regional cerebral blood flow (rCBF) changes with regional alterations of the blood-brain barrier (BBB) in various types of epileptiform seizures and in acute hypertension																										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 30%;">R. Suzuki</td> <td style="width: 40%;">Visiting Fellow</td> <td style="width: 20%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>C. Nitsch</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>K. Fujiwara</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>T. Kuroiwa</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>P. Ting</td> <td>Expert</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>I. Klatzo</td> <td>Chief, Lab. Neuropath. Neuroanat. Sci.</td> <td>LNNS NINCDS</td> </tr> </table>			PI:	R. Suzuki	Visiting Fellow	LNNS NINCDS	Other:	C. Nitsch	Visiting Fellow	LNNS NINCDS		K. Fujiwara	Visiting Fellow	LNNS NINCDS		T. Kuroiwa	Visiting Fellow	LNNS NINCDS		P. Ting	Expert	LNNS NINCDS		I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS
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Other:	C. Nitsch	Visiting Fellow	LNNS NINCDS																							
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	I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS																							
COOPERATING UNITS (if any)  None																										
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences																										
SECTION Section on Cerebrovascular Pathology																										
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																										
TOTAL MANYEARS: <div style="text-align: center; font-size: 1.2em;">2.0</div>	PROFESSIONAL: <div style="text-align: center; font-size: 1.2em;">1.8</div>	OTHER: <div style="text-align: center; font-size: 1.2em;">0.2</div>																								
CHECK APPROPRIATE BOX(ES)  <div style="display: flex; justify-content: space-between;"> <span><input type="checkbox"/> (a) HUMAN SUBJECTS</span> <span><input type="checkbox"/> (b) HUMAN TISSUES</span> <span><input checked="" type="checkbox"/> (c) NEITHER</span> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <span><input type="checkbox"/> (a1) MINORS</span> <span><input type="checkbox"/> (a2) INTERVIEWS</span> </div>																										
SUMMARY OF WORK (200 words or less - underline keywords)  Local BBB changes in drug-induced seizures and in acute hypertension were correlated in the same animals with rCBF changes. Our findings indicate that BBB changes are associated with marked regional elevations of the CBF. However, this relationship is relative and it does not indicate existence of any absolute threshold for rCBF elevation to be associated with BBB breakdown. This project is completed.																										

## Project Description:

Objectives: A regional character of epileptogenic activity induced by various agents provides a special opportunity to evaluate and compare several parameters most pertinent to the pathophysiology of seizures and to the mechanisms of blood-brain barrier (BBB) dysfunction. The metabolic changes associated with seizure activity obviously influence the regional cerebral blood flow (rCBF) changes. To what extent these rCBF changes are related to observed regional BBB disturbances and to systemic hypertension was the subject of this investigation.

Methods Employed: Twenty-nine New Zealand white rabbits were used in this study. There were 3 groups of animals. Group 1. Controls. Six rabbits were injected with 2 ml of normal saline through the ear vein. Group 2. Drug-induced seizures. Six rabbits were injected with 2 ml of saline containing 1.2 mg of bicucullin through the ear vein. The onset of seizure activity was determined by electroencephalogram (EEG). Group 3. Hypertension was induced by metaraminol (Aramine) in 4 rabbits or by adrenaline (0.5 mg) in some animals, preceded by injection of 0.2 mg of atropine.

The rCBF was evaluated by  $^3\text{H}$ -nicotine bitartrate injected through a femoral vein catheter at a constant rate for 40 seconds. Arterial blood samples were collected from a brachial arterial catheter every 2 seconds during nicotine infusion. The animals were decapitated 40 seconds after start of the  $^3\text{H}$ -nicotine infusion. The brains were quickly removed and dissected, and tissue samples were taken from 30 different brain regions. For evaluation of the BBB changes, the animals were injected with Evans Blue (EB) 30 minutes prior to experimentation.

Major Findings: The rCBF in the control group showed in each region, respectively, constant values, and there were no BBB changes. The bicucullin group showed significantly increased rCBF in all the regions and especially in the globus pallidus (538%), periaqueductal gray (533%), rhombencephalon (484%) and in a few other areas. These regions showed also definite extravasations of EB.

In acute hypertension induced by adrenaline, the highest rCBF elevations were observed in occipital cortex (239%) and in superior colliculus (236%) which also revealed BBB changes. The rCBF elevations in aramine-injected animals were lower and there was no evidence of EB leakage.

Significance to Biomedical Research and the Program of the Institute: Correlation of rCBF changes in epileptiform seizures and in acute hypertension with regional BBB changes observed in these conditions revealed that there is a certain relationship between rises in rCBF and the onset of BBB breakdown. In bicucullin convulsions and in adrenaline-induced hypertension, the appearance and localization of EB leakage correlated with the highest rCBF elevations observed in these animals. On the other hand, it was also evident that the level of rCBF elevation per se was not crucial since the BBB breakdown occurred at

quite different levels in these different conditions. Elucidation of factors which are actually triggering off the breakdown of the BBB should be of importance in understanding pathomechanisms of epileptic seizures. Our observations indicate that local tissue factors such as changes in neurotransmitter reactions may play a more significant role than purely vascular changes related to loss of autoregulation combined with an increased intravascular pressure.

Proposed Course of the Project: This project is completed and a major report on these studies is in preparation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02460-01 LNNS																				
PERIOD COVERED October 1, 1980 to September 30, 1981																						
TITLE OF PROJECT (80 characters or less) Interrelationship between the regional cerebral blood flow(rCBF), edematous changes in water content and vascular permeability in cerebral ischemia and in cryogenic brain edema																						
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																						
<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 60%;">I. Klatzo</td> <td style="width: 30%;">Chief, Lab. Neuropath. Neuroanat. Sci.</td> <td style="width: 10%;">LNNS NINCDS</td> </tr> <tr> <td rowspan="5">Other:</td> <td>F. Orzi</td> <td>Visiting Fellow</td> <td>LCM NIMH</td> </tr> <tr> <td>F. Schuier</td> <td>Guest Worker</td> <td>LCM NIMH</td> </tr> <tr> <td>H. Laursen</td> <td>Visiting Associate</td> <td>LNNS NINCDS</td> </tr> <tr> <td>R. Suzuki</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td>C. Nitsch</td> <td>Visiting Scientist</td> <td>LNNS NINCDS</td> </tr> </table>			PI:	I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS	Other:	F. Orzi	Visiting Fellow	LCM NIMH	F. Schuier	Guest Worker	LCM NIMH	H. Laursen	Visiting Associate	LNNS NINCDS	R. Suzuki	Visiting Fellow	LNNS NINCDS	C. Nitsch	Visiting Scientist	LNNS NINCDS
PI:	I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS																			
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	R. Suzuki	Visiting Fellow	LNNS NINCDS																			
	C. Nitsch	Visiting Scientist	LNNS NINCDS																			
COOPERATING UNITS (if any) Laboratory of Cerebral Metabolism, National Institute of Mental Health																						
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences																						
SECTION Section on Cerebrovascular Pathology																						
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																						
TOTAL MANYEARS: 2.1	PROFESSIONAL: 1.1	OTHER: 1.0																				
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS         </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div> <div style="display: flex; justify-content: space-between; align-items: flex-start; margin-top: 5px;"> <div> <input type="checkbox"/> (a1) MINORS         </div> <div> <input type="checkbox"/> (a2) INTERVIEWS         </div> </div>																						
SUMMARY OF WORK (200 words or less - underline keywords)  Evaluation of correlations between edematous, BBB changes and rCBF assessed by the quantitative method indicated a close synergistic relationship between ischemia and edema, one aggravating the other. The interesting finding in this study is that after complete healing of ischemic lesion there may remain a significant reduction of CBF in the affected hemisphere detectable 1 month after MCA occlusion. This project is completed.																						

## Project Description:

Objectives: Various observations suggest a close synergistic interrelationship between cerebral ischemia and edema. The purpose of this study was to investigate the extent of this interrelationship in order to understand better the pathophysiology of these conditions.

Methods Employed: Thirty-six cats were subjected to a permanent occlusion of the left middle cerebral artery (MCA) by transorbital approach. The rCBF was assessed by autoradiographic quantitative method of Sokoloff using  $^{14}\text{C}$  iodoantipyrine. The changes in water content were determined by a sensitive, newly standardized specific gravity method and changes in permeability of the BBB were evaluated with Evans Blue (EB) tracer or by application of immunocytochemical peroxidase-antiperoxidase (PAP) procedure designed to demonstrate extravasation of serum proteins. The cats were sacrificed in groups after 1 hour, 3, 6, 12, 24 hours, 2, 4 days, 1, 3 and 5 weeks. The cryogenic cortical lesion was produced in 4 animals which were sacrificed 24 hours later. All the above-mentioned parameters were assessed also in these animals.

Major Findings: The severe rCBF changes were demonstrable in animals sacrificed 1 hour after occlusion. Frequently at the periphery of regions with rCBF below 20 ml/100 g/l min there were hyperemic zones in which the PAP method revealed serum protein extravasations. Such hyperemic zones, sometimes with very high rCBF values, were demonstrable in animals sacrificed up to 4 days after occlusion. The extravasated proteins appeared to spread quickly and preferentially through the ischemically affected gray matter. The edematous changes, as judged by specific gravity (SG) measurements, progressed to reach their peak after 12-24 hours in the gray matter and after 2-4 days in the white matter. The edematous changes in the white matter were to some extent correlated with a migration of extravasated serum proteins from the gray into the white matter. At the same time, presumably due to necrotic digestion, the proteins were no longer visible in the gray matter with the PAP method. The leakage of EB was evident in cats sacrificed after 6 hours and the blue discoloration remained visible predominantly in the gray matter even in animals sacrificed weeks after occlusion. Besides sharply localized drastic reductions in the rCBF (below 20 ml/100g/l min), there was generalized moderate reduction in rCBF in the whole ipsilateral hemisphere observed as late as 1 month after MCA occlusion.

Cats subjected to cold lesion and sacrificed after 1 day showed a slight but significant reduction in the rCBF in gray matter regions, such as caudate and cerebral cortex, relatively distant from edema, which according to SG measurements showed no evidence of edema.

Significance to Biomedical Research and the Program of the Institute: Elucidation of interrelationships between edematous, BBB permeability changes and the precise level of ischemia, as determined by the quantitative rCBF assays, would be of value for interpretation of pathophysiology of ischemic lesions.



Our observations indicate that initially ischemic edema is of purely cytotoxic character and develops in areas below a certain threshold of rCBF. Changes of the BBB, allowing escape of serum proteins, introduce a vasogenic component of ischemic edema which is not dependent on severe reduction in rCBF values. On the other hand, vasogenic edema, presumably, by comparison of microcirculation, may produce a secondary reduction in rCBF even in relatively distant regions.

Proposed Course of the Project: This project is completed.

Publications:

I. Klatzo, F. Schuier, F. Orzi, F. Wilmes, F., Chui, E., Suzuki, R., Fujiwara, K., Nitsch, C., Laursen, H., Fenton, I., and Goping, G.: Inter-relationship between cerebral blood flow (CBF) and brain edema (BE). In: Proceedings of the Symposium on Cerebral Blood Flow. Foundation for Higher Medical Education of Netherlands Antilles, Curacao, October 1980. Clin. Neurol. Neurosurg. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02461-01 LNNS														
PERIOD COVERED October 1, 1980 to September 30, 1981																
TITLE OF PROJECT (80 characters or less) Factors in the reproducibility of the gravimetric method for evaluation of the edematous changes in the brain																
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 40%;">K. Fujiwara</td> <td style="width: 40%;">Visiting Fellow</td> <td style="width: 10%;">LNNS NINCDS</td> </tr> <tr> <td rowspan="3">Other:</td> <td>C. Nitsch</td> <td>Visiting Scientist</td> <td>LNNS NINCDS</td> </tr> <tr> <td>R. Suzuki</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td>I. Klatzo</td> <td>Chief, Lab. Neuropath. Neuroanat. Sci.</td> <td>LNNS NINCDS</td> </tr> </table>			PI:	K. Fujiwara	Visiting Fellow	LNNS NINCDS	Other:	C. Nitsch	Visiting Scientist	LNNS NINCDS	R. Suzuki	Visiting Fellow	LNNS NINCDS	I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS
PI:	K. Fujiwara	Visiting Fellow	LNNS NINCDS													
Other:	C. Nitsch	Visiting Scientist	LNNS NINCDS													
	R. Suzuki	Visiting Fellow	LNNS NINCDS													
	I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS													
COOPERATING UNITS (if any)  None																
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences																
SECTION Section on Cerebrovascular Pathology																
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																
TOTAL MANYEARS:	0.6	PROFESSIONAL: 0.5      OTHER: 0.1														
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																
SUMMARY OF WORK (200 words or less - underline keywords)  Factors influencing the reproducibility of the grayimetric method for regional assessment of edematous changes in the brain have been elucidated. This allowed a standardization of the method by strict control of the purity of basic ingredients of the column, the time of measurements, the size of tissue samples, and the temperature. With this method, highly reproducible and reliable measurements were obtained on the normal and/or the ischemically injured brain tissue. This project is completed.																

## Project Description:

Objectives: The specific gravity (SG) method, which is 5 to 10 times more sensitive than determination of water content by wet/dry weight ratios, is the most suitable approach for mapping out regional water content changes in the brain tissue, allowing thus an insight into the dynamics of edema development and resolution. Elucidation of the factors which could influence the reproducibility and reliability of this method is of great importance and was the subject of these investigations.

Methods Employed: The factors to be evaluated were: the purity of kerosene (K) and of monobromobenzene (MBB) - two basic ingredients of the gravimetric column, 2) duration of interaction of K and MBB with tissue samples and/or standard solutions, 3) the size of the tissue samples, and 4) the temperature. The corresponding determinations were carried out on the cerebral cortex of normal gerbils and of those subjected to cerebral ischemia by the unilateral occlusion of the common carotid artery.

Major Findings: With regard to the purity of K and MBB ingredients, the assays revealed that frequently, and especially the K, may be contaminated by water-soluble substances. Water treatment of the K and MBB removes the contaminant, and thus essentially contributes to reproducibility of the method. It was also shown that the size of tissue samples, the time the tissue samples remain in the column, and the temperature at which gravimetric measurements are carried out influence the results. With the standardization of the method especially with regard to purification of the column ingredients, time of measurement, size of tissue samples, and the temperature, most reproducible results were obtained on the normal brain tissue and that affected by ischemia.

Significance to Biomedical Research and the Program of the Institute: Elucidation of factors which influence the measurements of water content by the gravimetric method allowed a strict standardization of this method. The gravimetric method allows a quick and reliable regional assessment of edematous changes and thus it is of great value in our studies on the pathomechanisms of brain edema.

Proposed Course of the Project: This project is completed.

## Publications:

Fujiwara, K., Nitsch, C., Suzuki, R., and Klatzo, I.: Factors in the reproducibility of the gravimetric method for evaluation of edematous changes in the brain. Neurol. Res. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 01999-09 LNNS
PERIOD COVERED October 1, 1980, to September 30, 1981		
TITLE OF PROJECT (80 characters or less) Transport studies in ischemic cerebral edema		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: M. Spatz                      Head, Section on Neurocytobiology                      LNNS NINCDS		
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences		
SECTION Section on Neurocytobiology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:                      0	PROFESSIONAL:                      0	OTHER:                      0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  This project has been temporarily discontinued.		

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02000-09 LNNS												
PERIOD COVERED October 1, 1980 to September 30, 1981														
TITLE OF PROJECT (80 characters or less) Biochemistry of brain edema in cerebral ischemia of gerbils														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 20%;">M. Spatz</td> <td style="width: 50%;">Head, Section on Neurocytobiology</td> <td style="width: 20%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>K. Abe</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>I. Klatzo</td> <td>Chief, Lab. Neuropath. Neuroanat. Sci.</td> <td>LNNS NINCDS</td> </tr> </table>			PI:	M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS	Other:	K. Abe	Visiting Fellow	LNNS NINCDS		I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS
PI:	M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS											
Other:	K. Abe	Visiting Fellow	LNNS NINCDS											
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COOPERATING UNITS (if any) None														
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences														
SECTION Section on Neurocytobiology														
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205														
TOTAL MANYEARS: <div style="text-align: center;">0</div>	PROFESSIONAL: <div style="text-align: center;">0</div>	OTHER: <div style="text-align: center;">0</div>												
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;"><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td style="width: 33%;"><input type="checkbox"/> (b) HUMAN TISSUES</td> <td style="width: 33%;"><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td colspan="3"><input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS</td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS								
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<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS														
SUMMARY OF WORK (200 words or less - underline keywords)  This project has been terminated.														



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02165-07 LNNS									
PERIOD COVERED October 1, 1980, to September 30, 1981											
TITLE OF PROJECT (80 characters or less) Correlation of <sup>3</sup> H isoleucine uptake in pia arachnoid with culture of fibro- blasts											
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table> <tr> <td>PI: M. Spatz</td> <td>Head, Section on Neurocytobiology</td> <td>LNNS NINCDS</td> </tr> <tr> <td>Other: M. R. Murray</td> <td>Research Biologist</td> <td>LNNS NINCDS</td> </tr> <tr> <td>I. Klatzo</td> <td>Chief, Lab. Neuropath. Neuroanat. Sci.</td> <td>LNNS NINCDS</td> </tr> </table>			PI: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS	Other: M. R. Murray	Research Biologist	LNNS NINCDS	I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS
PI: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS									
Other: M. R. Murray	Research Biologist	LNNS NINCDS									
I. Klatzo	Chief, Lab. Neuropath. Neuroanat. Sci.	LNNS NINCDS									
COOPERATING UNITS (if any) None											
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences											
SECTION Section on Neurocytobiology											
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205											
TOTAL MANYEARS: 0	PROFESSIONAL: 0	OTHER: 0									
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS											
SUMMARY OF WORK (200 words or less - underline keywords)  This project has been terminated.											

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02275-05 LNNS						
PERIOD COVERED October 1, 1980 to September 30, 1981								
TITLE OF PROJECT (80 characters or less) Cerebral capillary endothelial cultures								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%;"> <tr> <td style="width: 33%;">PI: M. Spatz</td> <td style="width: 33%;">Head, Section on Neurocytobiology</td> <td style="width: 33%;">LNNS NINCDS</td> </tr> <tr> <td>Other: M. Murray</td> <td>Research Biologist</td> <td>LNNS NINCDS</td> </tr> </table>			PI: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS	Other: M. Murray	Research Biologist	LNNS NINCDS
PI: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS						
Other: M. Murray	Research Biologist	LNNS NINCDS						
COOPERATING UNITS (if any) Dr. Lawrence DeBault, Department of Pathology, Children's Hospital, Oklahoma City, Oklahoma								
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences								
SECTION Section on Neurocytobiology								
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205								
TOTAL MANYEARS: <div style="text-align: center;">1.0</div>	PROFESSIONAL: <div style="text-align: center;">0.5</div>	OTHER: <div style="text-align: center;">0.5</div>						
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS         </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div> <input type="checkbox"/> (a1) MINORS         </div> <div> <input type="checkbox"/> (a2) INTERVIEWS         </div> </div>								
SUMMARY OF WORK (200 words or less - underline keywords) The established <u>cerebral endothelial cell cultures</u> derived from dissociated <u>cerebral microvessels</u> possess a specific endothelial plasma membrane antigen which provides a useful marker for the study of endothelium <u>in the living state</u> .								

## Project Description:

Objectives: The continuous study of the established cerebral endothelial cultures has been concerned with identification of specific endothelial plasma membrane antigen in endothelial cell cultures in order to obtain a reliable endothelial cell marker which would be useful for other studies of endothelium in the living state.

Methods Employed: The endothelial cell culture was derived from dissociated cells of rat's cerebral capillaries by the method of Spatz et al. (Brain Res. 191: 577-582, 1980). Acetone-fixed cover slip culture or frozen sections of intact tissue (brain and liver) were incubated with rabbit anti ME-2 cell membrane (R Ant ME-2 mem; this antiserum was previously prepared in rabbits against the membrane fraction of mouse endothelial cell line, ME-2 derived from cerebral microvessels - DeBault et al., in press). Control specimens were treated with normal rabbit serum (NRS) or PBS. After washing, the preparations were incubated with fluorescein-conjugated goat anti-rabbit immunoglobulin antiserum for 30 minutes at 37°C, washed and mounted.

Major Findings: The indirect immunofluorescence studies on frozen sections of rat brain showed good cross reactivity and specificity for endothelial cell only (the neuroglial was negative). The endothelial cell culture displayed a specific diffuse surface fluorescence, a pattern similar to that seen with ME-2 mouse endothelial cells. A strong mitochondrial fluorescence was also observed but it was shown to be, in part, nonspecific by NRS and PBS controls. This fluorescence is most probably due to mitochondrial membrane cross reactivity with a component of the antiserum. The cell-type-specific fluorescence (diffuse surface fluorescence) present in the cultured cells is indicative of an endothelial antigen presence.

Significance for Biomedical Research and the Program of the Institute: The established cerebral capillary endothelial cell cultures provide a pure cell line which will be useful for the investigation of cerebral endothelial cells in the living state without the influence of any other cells. Thus, the function of cerebral capillary endothelium as compared to endothelium derived from capillaries outside the blood-brain barrier (BBB) system can be characterized under normal and pathologic conditions. This approach will also add another dimension for the studies related to the BBB permeability in various disease processes.

Proposed Course of the Project: The primary objective of this project has been to characterize further the endothelial cell line in order to obtain a reliable endothelial marker of the study of the mechanism involved in their unique function as constituents of the BBB.

## Publications:

Spatz, M., and Mrsulja, B. B.: Progress in cerebral microvascular studies related to the function of BBB. In: Advances in Cellular Neurobiology. New York, Academic Press (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02324-04 LNNS						
PERIOD COVERED October 1, 1980 to September 30, 1981								
TITLE OF PROJECT (80 characters or less) Studies on the blood-brain barrier (BBB) to 5-hydroxytryptamine and norepi- nephine metabolites Former title: Studies on the blood-brain barrier (BBB) to 5-hydroxytryptamine								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%;"> <tr> <td style="width: 33%;">PI: M. Spatz</td> <td style="width: 33%;">Head, Section on Neurocytobiology</td> <td style="width: 33%;">LNNS NINCDS</td> </tr> <tr> <td>Other: C. Maruki</td> <td>Visiting Fellow</td> <td>LNNS NINCOS</td> </tr> </table>			PI: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS	Other: C. Maruki	Visiting Fellow	LNNS NINCOS
PI: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS						
Other: C. Maruki	Visiting Fellow	LNNS NINCOS						
COOPERATING UNITS (if any) None								
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences								
SECTION Section on Neurocytobiology								
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205								
TOTAL MANYEARS: 0.6	PROFESSIONAL: 0.3	OTHER: 0.3						
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS								
SUMMARY OF WORK (200 words or less - underline keywords) In contrast to the specific capillary uptake of 5-hydroxytryptamine (5-HT) and norepinephrine (NE), the uptake of 5-hydroxyindole-3-acetic acid (5-HIAA), normetanephrine (NM), and metanephrine (ME) was found to be nonspecific in the isolated microvessels.								

## Project Description:

**Objectives:** Previously we have demonstrated that the isolated cerebral microvessels are capable of taking up norepinephrine (NE) and its non-metabolizable analogue metaraminol (M) as well as 5-hydroxytryptamine (5-HT) by  $\text{Na}^+$ - and  $\text{K}^+$ -dependent, carrier mediated process (Abe et al., in Advances in Experimental Biology and Medicine, Vol. 131. The Cerebral Microvasculature, New York, Plenum, 1980, pp. 45-55). To elucidate further the intrinsic function of these vessels in regard to blood-brain barrier (BBB) to monoamines, we investigated the characteristics of capillary uptake of NE and 5-HT metabolites.

**Methods Employed:** The isolated cerebral microvessels were incubated either with  $^3\text{H}$  normetanephrine (NM) or  $^3\text{H}$  metanephrine (ME) or  $^{14}\text{C}$  5-HIAA in Ringer solution containing .1% albumin (pH 7.4) alone or with various concentrations of unlabeled substrates. The influence of metabolic inhibitors, amines, amino acids and bivalent ions was also tested by the addition of a given substrate to the incubating medium containing the labeled compound. The level of radioactivity in the samples was counted in a liquid scintillation counter, and the proteins were determined by Lowry's technique.

**Major Findings:** In contrast to NE and 5-HT, the capillary uptake of NM, ME and 5-HIAA could not be influenced either by the addition of their respective unlabeled substrate or other catechols, amino acids, hypothermia or the duration of the incubating time (30 sec - 15 min:  $^3\text{H}$  NM 41-55 nmoles/mg/prot;  $^3\text{H}$  ME 91-100 nmoles/mg/prot;  $^{14}\text{C}$  HIAA 11.8-13.4  $\mu\text{moles/mg/prot}$ ). Bivalent ions increased the uptake of NM and that of ME slightly but had no effect on 5-HIAA. The metabolic inhibitors (Na azide, DNP, NaF and KCN) had no effect on the capillary uptake of NM, ME and 5-HIAA.

**Significance to Biomedical Research and the Program of the Institute:** These results indicate that the limited uptake of NE and 5-HT metabolites in cerebral microvessels is nonspecific since it could neither be influenced by self- or cross-inhibition or by hypothermia. These findings together with previously observed time-dependent accumulation of NE and 5-HT metabolites in the incubating medium when NE or 5-HT was incubated with the cerebral microvessels, indicate that the capillary vessels are incapable of accumulating these metabolites.

**Proposed Course of the Project:** These investigations have been extended to other members of the catecholamine family. Part of the work was presented at the Neuroscience Meeting in Cincinnati, November 1980, and part was presented at the Symposium on Cerebral Microcirculation and Metabolism in Berlin, July 1980. Project Z01 NS 02358-03 LNNS has been incorporated into this project.



Publications: See Z01 NS 02360-03 LNNS

Spatz, M., Maruki, C., Abe, T., Rausch, W. D., Abe, K., and Merkel, N.: The uptake and fate of the radiolabeled 5-hydroxytryptamine in isolated cerebral microvessels. Brain Res. 217 (in press).

Spatz, M., Abe, T., Rausch, W. D., Abe, K., Merkel, N., and Maruki, C.: Studies on the nature and function of cerebral microvessel involvement in the blood-brain barrier for monoamines. In Cervaeos-Navarro, J., and Fritschka, E. (Eds.): Cerebral Microcirculation and Metabolism. New York, Raven Press, 1981, pp. 23-28.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02327-04 LNNS						
PERIOD COVERED October 1, 1980 to September 30, 1981								
TITLE OF PROJECT (80 characters or less)  The study of monoamines' uptake and pinocytotic activity of pia arachnoid cultures.								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: M. Spatz</td> <td style="width: 33%;">Head, Section on Neurocytobiology</td> <td style="width: 33%;">LNNS NINCDS</td> </tr> <tr> <td>Other: M. R. Murray</td> <td>Research Biologist</td> <td>LNNS NINCDS</td> </tr> </table>			PI: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS	Other: M. R. Murray	Research Biologist	LNNS NINCDS
PI: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS						
Other: M. R. Murray	Research Biologist	LNNS NINCDS						
COOPERATING UNITS (if any) Dr. H. Hervonen, Department of Biomedical Sciences, University of Tampere, Tampere, Finland								
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences								
SECTION Section on Neurocytobiology								
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland								
TOTAL MANYEARS: 0.5	PROFESSIONAL: 0.3	OTHER: 0.2						
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS								
SUMMARY OF WORK (200 words or less - underline keywords)  The pia arachnoid membrane as a constituent of the blood-brain barrier (BBB) has been cultivated and evaluated in regard to the <u>monoamine uptake and pinocytotic activity</u> which are restricted by the BBB <u>in vivo</u> . Both cell types (endothelium and pia) of the pia arachnoid membrane showed a limited uptake of catecholamines and serotonin. L-dopa was taken up by the endothelial but not by the pial cells while the reverse situation was observed in regard to the pinocytotic activity.								

## Project Description:

Objectives: Pia arachnoid and its blood vessels constitute one of the sites where the brain and cerebrospinal fluid (CSF) are separated from the blood and external tissue by so-called blood-brain barrier (BBB) (Rapoport, S., Blood-brain Barrier in Physiology and Medicine, Raven Press, N.Y., 1976).

The aim of this study has been to evaluate the characteristics of pia arachnoid cellular components toward biogenic amines and pinocytotic activity which are restricted by the BBB in vivo.

Methods Employed: The pia arachnoid membrane was prepared from newborn rats and cultured on glass in a Maximow double coverslip assembly for 2-3 weeks.

The incubations for the catecholamine uptake were performed in a Hepes-buffered (20 mM) Locke's salt solution, pH 7.4, at room temperature. The cultures were first briefly washed to remove the culture medium, then preincubated for 10 minutes with or without pargyline (a monoamine oxidase inhibitor) and pyrogallol (a catechol-O-methyl transferase inhibitor). The incubation time was 10 minutes, again with or without pargyline and pyrogallol according to the preincubation. The following biogenic amines and precursors were used in  $10^{-5}$  to  $10^{-6}$  M concentrations: L-dopa, dopamine, noradrenaline, adrenaline and serotonin. After incubation the cultures were washed in Hepes-Locke's solution for 5 seconds to 10 minutes before processing for either formaldehyde-induced fluorescence or glyoxylic acid-induced fluorescence.

Zeiss Axiomat microscope was used to observe the fluorescence operating either with transmitted light with EG 12 excitation filter, dark-field condensor and LP 500 barrier filter or with epi-illumination with BG 12 and BP 405 excitation filters, LP 470 barrier filter and a dichroic mirror. The same microscope was used for phase-contrast microscopy. Unstained or stained (uranyl acetate and lead citrate) thin sections were examined with Philips EM 300 and JEOL 100C electron microscopes.

Major Findings: Two populations of cells have been identified in the cultures of the pia arachnoid membrane by both light and electron microscopy: 1) endothelial cells and (2) pia arachnoid cells. Specific L-dopa uptake and accumulation of biogenic amines were demonstrated with glyoxylic acid histochemistry in the endothelial cells but not in the pia arachnoid cells. Uptake of the monoamines was of extraneuronal type (Iversen, L.L., *Brit. J. Pharmacol. Chemotherap.* 25, 18-33, 1965) and was found to be equally limited in both cell types. The pia arachnoid cells show a high pinocytotic activity by removing the horseradish peroxidase from the incubating medium. However, the endothelial cells did not display any signs of pinocytosis or vesicular transport showing virtually no intracellular horseradish peroxidase.

Significance to Biomedical Research and the Program of the Institute:

The pia arachnoid offers a relatively simple in vitro model for the study of factors influencing pinocytosis and the vesicular transport in the cellular constituents of the BBB which are altered in many pathological conditions.

Proposed Course of the Project: This part of the investigation of pia-arachnoid has been completed and published in Brain Research.

Further studies have been undertaken to localize the site of the monoamines' uptake by cytoimmunochemistry.

Publications:

Hervonen, H., Spatz, M., Bembry, J., and Murray, M. R.: Studies related to the blood-brain barrier to monoamines and proteins in pia-arachnoid cultures. Brain Res. 210: 449-454, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02328-04 LNNS						
PERIOD COVERED October 1, 1980 to September 30, 1981								
TITLE OF PROJECT (80 characters or less) The effect of cholinesterase inhibitors on nerve cells developing in cultures of spinal ganglia. Former title: The effect of cholinesterase inhibitors on nerve cells developing in cultures of spinal and sympathetic ganglia								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%;"> <tr> <td style="width: 33%;">PI: M. R. Murray</td> <td style="width: 33%;">Research Biologist</td> <td style="width: 33%;">LNNS NINCDS</td> </tr> <tr> <td>Other: M. Spatz</td> <td>Head, Section on Neurocytobiology</td> <td>LNNS NINCDS</td> </tr> </table>			PI: M. R. Murray	Research Biologist	LNNS NINCDS	Other: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS
PI: M. R. Murray	Research Biologist	LNNS NINCDS						
Other: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS						
COOPERATING UNITS (if any) Dr. H. Hervonen, Department of Biomedical Sciences, University of Tampere, Tampere, Finland								
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences								
SECTION Section on Neurocytobiology								
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205								
TOTAL MANYEARS: <div style="text-align: center;">0.3</div>	PROFESSIONAL: <div style="text-align: center;">0.2</div>	OTHER: <div style="text-align: center;">0.1</div>						
CHECK APPROPRIATE BOX(ES) <table style="width: 100%;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td colspan="3"> <input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS           </td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER						
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS								
SUMMARY OF WORK (200 words or less - underline keywords)  The <u>inhibition of cholinesterases</u> , especially <u>acetylcholinesterase</u> , in the <u>developing neuroblast of the spinal and sympathetic ganglion</u> leads to a <u>growth inhibition and degeneration of the neurons</u> , strongly supporting the <u>hypothesis that acetylcholinesterase plays an important role in the maturation of the neurons</u> . This investigation has been completed and a manuscript is in preparation.								



Project Description:

Objectives: It is known that acetylcholinesterase (AChE) appears early in developing neurons before the onset of cholinergic transmission and it was thought that the sensory ganglia might provide some crucial information in this matter since these are neither cholinergic nor cholinceptive. The purpose of this work was to inquire into the possible role of cholinesterase in the general maturation of neurons aside from their accepted enzymatic functions in neurotransmission.

Methods Employed: The spinal and sympathetic ganglia were prepared from 8-day-old chick embryos and cultured in Maximow double coverslip assembly up to 4 weeks *in vitro*. The specific and nonspecific AChE inhibitors ( eserine, iso-OMPA, BW 274C51, DFP and paraoxon) in concentration  $10^{-7}$  to  $10^{-3}$  M were added either for the entire culture period or for a 30 minutes to 3 days exposure to nutrient medium. Observations in the perikaryal morphology and size (growth and differentiation) as well as fiber outgrowth were made on the living and stained ganglia (total of 242 cultures) at successive stages in their development by light and electron microscopy.

Major Findings: There was a distinct difference in the neuronal development of the dorsal root ganglia when cultured with or without the cholinesterase inhibitors. At low concentrations ( $10^{-7}$  -  $10^{-5}$  M) in the cultures the inhibitors affected the growth and maturation of the neurons. They were small with slightly irregularly outlined nuclei showing a fully dispersed chromatin and a poorly developed nucleolus. Large amounts of free ribosomes but little of rough surfaced endoplasmic reticulum (RER) and few abnormal mitochondria were seen in the cytoplasm.

The satellite cells surrounded the neurons completely, showed dark cytoplasm with sparse organelles, and a few lipid droplets. Less abnormalities were found with iso-OMPA, the nonspecific, than with the specific AChE inhibitors. Moreover, the short term treatment of the cultures with the AChE blocker was less effective and reversible since the cellular characteristics of the ganglia were found normal in 3-week-old cultures.

The same AChE inhibitors except for iso-OMPA when added to the culture medium at concentrations higher than  $10^{-5}$  M caused marked degeneration and necrosis of the spinal ganglia. From this it appears that AChE performs some function in neuron development which is not related to neurotransmission.

Significance to Biomedical Research and the Program of the Institute: The findings of this investigation strongly suggest that the AChE plays a significant role in neuronal development which is most likely unrelated to the neurotransmission. The significance of this study is to further explore the role(s) of an enzyme/a group of enzymes (acetylcholinesterase/cholinesterases) which have a widespread occurrence in the nervous system.

Proposed Course of the Project: This investigation has been completed and a manuscript is in preparation.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02357-03 LNNS						
PERIOD COVERED October 1, 1980 to September 30, 1981								
TITLE OF PROJECT (80 characters or less) The therapeutic $\gamma$ -hydroxybutyrate effect on experimental cerebral ischemia in Mongolian gerbils								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: M. Spatz</td> <td style="width: 33%;">Head, Section on Neurocytobiology</td> <td style="width: 33%;">LNNS NINCDS</td> </tr> <tr> <td>Other: C. Maruki</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> </table>			PI: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS	Other: C. Maruki	Visiting Fellow	LNNS NINCDS
PI: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS						
Other: C. Maruki	Visiting Fellow	LNNS NINCDS						
COOPERATING UNITS (if any)  None								
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences								
SECTION Section on Neurocytobiology								
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205								
TOTAL MANYEARS: 0.8	PROFESSIONAL: 0.4	OTHER: 0.4						
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input type="checkbox"/> (b) HUMAN TISSUES</td> <td><input checked="" type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td><input type="checkbox"/> (a1) MINORS</td> <td><input type="checkbox"/> (a2) INTERVIEWS</td> <td></td> </tr> </table>			<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS	
<input type="checkbox"/> (a) HUMAN SUBJECTS	<input type="checkbox"/> (b) HUMAN TISSUES	<input checked="" type="checkbox"/> (c) NEITHER						
<input type="checkbox"/> (a1) MINORS	<input type="checkbox"/> (a2) INTERVIEWS							
SUMMARY OF WORK (200 words or less - underline keywords)  The ischemic reduction of the tyrosine and tryptophan activation in the brain has been prevented by the administration of $\gamma$ -hydroxybutyrate (GHB) prior to the bilateral carotid artery occlusion in gerbils.								

## Project Description:

**Objectives:** Recently we have shown that the naturally occurring central nervous system (CNS) depressants,  $\gamma$ -hydroxybutyrate (GHB) and its lactone  $\gamma$ -butyrolactone (GBL), modified cerebral ischemia as was manifested by amelioration of cerebral metabolites and edema changes occurring in the ischemic gerbils (Smialek, Klatzo and Spatz, in: Cerebral Vascular Disease 2, Excerpta Medica, 1979; Abe, Abe, Klatzo and Spatz, in: Advances in Neurology, Vol. 28. Brain Edema, Raven Press, 1980). In order to elucidate the mechanism of GHB or GBL action in ischemic brain, we investigated the enzymes involved in the synthesis and metabolism of neurotransmitters, some of which are affected by GHB or GBL under normal conditions (Walters and Roth, in: Neuroregulators and Psychiatric Disorders, Oxford University Press, 1977).

**Methods Employed:** Fifteen minutes of bilateral common carotid artery occlusion with and without release served as a model for the production of cerebral ischemia in gerbils. The treatment consisted of a single injection of GHB (500 mg/kg) 2 minutes prior to occlusion. Sham-operated and GHB-injected as well as untreated gerbils served as controls. Tyrosine hydroxylase, tryptophan hydroxylase and dopa decarboxylase were assayed by either radiolabeled or spectrofluorescent techniques (Nagatsu, T., Biochemistry of Catecholamines, University of Tokyo Press, 1973).

**Major Findings:** Bilateral carotid artery occlusion (15 min) without release had no effect on either the tyrosine or tryptophan activities in the gerbil's brain. However, animals subjected to 15 minutes' carotid clipping and 1, 4, 24 hrs clip release revealed a 33% lower activity of tyrosine hydroxylase in the basal ganglia and/or cerebral cortex than the GHB pretreated and sham-operated gerbils (normal and sham  $2.97 \pm .05$  nmoles/mg Protein/1 hr = 100%). The level of tryptophan hydroxylase activity was also found to be reduced to the same degree as that of tyrosine hydroxylase in the untreated animals but at a later date (48 hrs) after the reestablishment of the cerebral circulation. The GHB pretreatment prevented also the drop of this enzymatic activity. These findings suggest that GHB modulates the ischemic decrease of tyrosine and tryptophan hydroxylase activity and in this way affects the synthesis of biogenic amines.

**Significance to Biomedical Research and the Program of the Institute:** The beneficial therapeutic effect of the naturally occurring CNS depressants in the experimentally induced ischemia indicates that these substances might be useful clinically following the complete evaluation of these agents in various models of cerebral ischemia.

**Proposed Course of the Project:** To study of the effect of GHB on catecholamine and other metabolic pathways in the brain will be continued in cerebral ischemia in order to elucidate the pathophysiological mechanism of their beneficial action.

**Publications:** None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02358-03 LNNS												
PERIOD COVERED October 1, 1980 to September 30, 1981														
TITLE OF PROJECT (80 characters or less)  The postischemic effect on the uptake and metabolism of monoamines in isolated cerebral capillaries														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">T. Abe</td> <td style="width: 35%;">Visiting Fellow</td> <td style="width: 15%; text-align: right;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>K. Abe</td> <td>Visiting Fellow</td> <td style="text-align: right;">LNNS NINCDS</td> </tr> <tr> <td></td> <td>M. Spatz</td> <td>Head, Section on Neurocytobiology</td> <td style="text-align: right;">LNNS NINCDS</td> </tr> </table>			PI:	T. Abe	Visiting Fellow	LNNS NINCDS	Other:	K. Abe	Visiting Fellow	LNNS NINCDS		M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS
PI:	T. Abe	Visiting Fellow	LNNS NINCDS											
Other:	K. Abe	Visiting Fellow	LNNS NINCDS											
	M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS											
COOPERATING UNITS (if any)  B. B. Mrsulja, Institute of Biochemistry, Faculty of Medicine, Belgrade, Yugoslavia														
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences														
SECTION Section on Neurocytobiology														
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205														
TOTAL MANYEARS: <div style="text-align: center;">0</div>	PROFESSIONAL: <div style="text-align: center;">0</div>	OTHER: <div style="text-align: center;">0</div>												
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS														
SUMMARY OF WORK (200 words or less - underline keywords)  This project has been combined with Project Z01 NS 02324-04 LNNS.														

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02360-03 LNNS
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less) The effect of central nervous system depressants on ischemic cerebral edema of gerbils		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: M. Spatz Head, Section on Neurocytobiology LNNS NINCDS		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences		
SECTION Section on Neurocytobiology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.2	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  This project is completed and resulted in two publications:  Spatz, M., Abe, K., Smialek, M., and Abe, T.: Evaluation of gamma-hydroxybutyrate treatment in experimental cerebral ischemia. In Betz, E., Grote, J., Heuser, D., and Wüllenweber, R. (Eds.): <u>Pathophysiology and Pharmacotherapy of Cerebrovascular Disorders</u> , Baden-Baden-Köln-New York, Verlag Gerhard Witzstock, 1980, pp. 286-289.  Abe, K., Abe, T., and Spatz, M.: The effect of endogenous and exogenous central nervous system depressants on ischemic edema. In: <u>10th Salzburg Conference on Cerebral Vascular Disease</u> , September 1980 (in press).		



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02361-04 LNNS						
PERIOD COVERED October 1, 1980 to September 30, 1981								
TITLE OF PROJECT (80 characters or less) Investigations on blood-brain barrier (BBB) permeability								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%;"> <tr> <td style="width: 33%;">PI: M. Spatz</td> <td style="width: 33%;">Head, Section on Neurocytobiology</td> <td style="width: 33%;">LNNS NINCDS</td> </tr> <tr> <td>Other: C. Maruki</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> </table>			PI: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS	Other: C. Maruki	Visiting Fellow	LNNS NINCDS
PI: M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS						
Other: C. Maruki	Visiting Fellow	LNNS NINCDS						
COOPERATING UNITS (if any) None								
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences								
SECTION Section on Neurocytobiology								
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205								
TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.3	OTHER: 0						
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS								
SUMMARY OF WORK (200 words or less - underline keywords) The permeability of the blood-brain barrier has been evaluated in gerbils subjected to bilateral <u>cerebral ischemia and postischemia</u> . The BBB permeability was found to be selectively altered in the postischemic period but not during cerebral ischemia.								

## Project Description:

Objectives: The aim of this study has been to investigate the permeability of the blood-brain barrier (BBB) in bilateral cerebral ischemia, since unilateral ischemia produced selective and diverse effects of BBB functions in the affected cerebral hemisphere (Spatz, Fujimoto, Go, in Dynamics of Brain Edema, Berlin-Heidelberg, Springer Verlag, pp. 181-186, 1976).

Methods Employed: Several groups of adult gerbils were subjected to bilateral common carotid artery clipping for 3, 6 and 15 minutes with and without clip release. The following tracers have been used so far for the evaluation of the BBB: NaFl, Evans blue,  $^{14}\text{C}$  sucrose,  $^3\text{H}$  norepinephrine (NE),  $^3\text{H}$  5-hydroxytryptamine (5-HT) and  $^3\text{H}$  dextran (Mol. weight 60,000).

Major Findings: The BBB permeability was found to be intact to NaFl, sucrose and Evans blue during the 3, 6 and 15 minutes of bilateral common carotid artery occlusion. However, 30-50% of gerbils showed an increased BBB permeability to NaFl and sucrose after 30 minutes of reestablished cerebral circulation. The incidence of increased BBB permeability to NaFl and sucrose depended on the duration of ischemia and was not seen in animals with the clip released for 3 and 5 hours following occlusion for 3 and 6 minutes, respectively. In 15 minutes of bilateral occlusion, the greatest incidence of BBB sucrose leakage was seen after 3 and 7 days of recovery, while that of dextran was at 7 days of clip release. Moreover, selective BBB permeability changes to monoamines were observed in the recovery of 24 hours (5-HT) and 72 hours (NE).

Significance to Biomedical Research and the Program of the Institute: The basic comprehension of the blood-brain barrier behavior and function concerned with the passage of nutrient and non-nutrient substances from blood to brain following cerebral ischemia is of major importance (1) for the understanding of the mechanism responsible for the development of ischemic edema, as well as elucidating other pathophysiological processes in cerebrovascular disease and many other neurological disorders, and (2) for selecting the best therapeutic approach to a given disease.

Proposed Course of the Project: These investigations are still incomplete and require the evaluation of the BBB permeability changes and/or recovery at late periods of recirculation after 15 minutes of bilateral cerebral ischemia.

Publications: See Project No. Z01 NS 02324-04 LNNS

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02462-01 LNNS									
PERIOD COVERED October 1, 1980 to September 30, 1981											
TITLE OF PROJECT (80 characters or less) Biochemistry of brain ischemia and ischemic edema in Mongolian gerbils: $\beta$ -adrenergic receptor studies											
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT											
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: I. Karniouchina</td> <td style="width: 33%;">Visiting Fellow</td> <td style="width: 33%;">LNNS NINCDS</td> </tr> <tr> <td>Other: C. Maruki</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td>M. Spatz</td> <td>Head, Section on Neurocytobiology</td> <td>LNNS NINCDS</td> </tr> </table>			PI: I. Karniouchina	Visiting Fellow	LNNS NINCDS	Other: C. Maruki	Visiting Fellow	LNNS NINCDS	M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS
PI: I. Karniouchina	Visiting Fellow	LNNS NINCDS									
Other: C. Maruki	Visiting Fellow	LNNS NINCDS									
M. Spatz	Head, Section on Neurocytobiology	LNNS NINCDS									
COOPERATING UNITS (if any)  None											
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences											
SECTION Section on Neurocytobiology											
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205											
TOTAL MANYEARS: <div style="text-align: center;">1.1</div>	PROFESSIONAL: <div style="text-align: center;">1.0</div>	OTHER: <div style="text-align: center;">0.1</div>									
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS											
SUMMARY OF WORK (200 words or less - underline keywords)  The pathogenetic investigations of cerebral ischemia have been concerned with determining the effect of this process on the neurotransmitter receptors, in particular the $\beta$ -adrenergic type; these studies have shown that brain ischemia affects the membrane affinity for $^3\text{H}$ dihydroalprenolol ( $^3\text{H}$ -DHA) binding sites due to a decreased association but not dissociation rate of the ligand to the $\beta$ -receptors.											

## Project Description

**Objectives:** Cerebral ischemia had been shown to alter the levels of neurotransmitters and the activity of their metabolizing enzymes as well as the concentration of adenylate cyclase and cAMP in the brain. The changes in the cerebral biogenic amines and their degrading enzymes displayed an "oscillatory pattern" which could not be correlated to either the levels of the amines or to their catabolic enzymes. However, the observed behavior of these amines coincided with the periods of hyperactivity and hypoactivity of the brain in post-ischemia (Mrsulja et al., *Brain Res.* 119, 480-489, 1977; Cvejic et al., in Spatz, M., Mrsulja, B. B., Rakic, Lj. M., and W. D. Lust, Eds.: *Circulatory and Developmental Aspects of Brain Metabolism*, 1980, pp. 97-102). Since some of these processes could be either related to or a result of neurotransmitters receptor's changes we investigated the binding characteristics of  $\beta$ -adrenergic receptors in the ischemic model.

**Methods Employed:** 1. Cerebral ischemia was induced in Mongolian gerbils by bilateral common carotid artery clipping for 15 minutes and various periods of clip release. 2. Freshly dissected cerebral cortex and hippocampus were used (within 1 hour) to obtain the membrane  $P_2$  (50,000 g) fraction (by homogenization with 10 volumes of 0.32 M sucrose in 20 mM Tris-HCl, pH 7.4, and two differential centrifugations). The  $P_2$  fraction was washed in 30 volumes of 50 mM Tris-HCl buffer, pH 7.8, and centrifuged at 50,000 g for 10 minutes. 3. Binding studies were performed in 500  $\mu$ l duplicate samples containing about 5  $\mu$ g of membrane protein, 1 mM  $MgCl_2$ , a displacer for measuring the nonspecific binding and an appropriate concentration of  $^3H$  dihydroalprenolol ( $^3H$ -DHA) in 50 mM Tris-HCl buffer, pH 7.8. All samples were preincubated for 20 minutes at room temperature (for elimination of endogenous neurotransmitters) before adding the  $^3H$ -DHA ligand. After the sample incubation with  $^3H$ -DHA at the same temperature, the reaction was stopped by rapid filtration through GF/ $\beta$  filters and washed (3 x 4 ml) with ice-cold 50 mM Tris-HCl buffer, pH 7.8. The radioactivity of the samples on the filters mixed with aquasol was determined by counting the samples in a Beckman LS 9000 liquid scintillation spectrophotometer.

**Major Findings:** The membranous fraction ( $P_2$ ) obtained from normal gerbil's cerebral cortex and hippocampus displayed a specific (saturable) high affinity binding site for  $^3H$ -DHA with an apparent dissociation constant ( $K_D$ ) 1.0 mM and an apparent binding maximum ( $B_{max}$ ) of 136 pmol/mg protein. The Hill plot analysis revealed a single binding site since the slope was found to be close to 1.0. The preliminary analysis in regard to the membranous subtypes of  $\beta$ -receptors using the agonists and antagonists of norepinephrine (NE) showed the presence of both  $\beta_1$  and  $\beta_2$  adrenergic receptors but with a predominance of  $\beta_2$  receptors in the tissues.

Cerebral ischemia of 15 min duration led to a change in the affinity for  $^3H$ -DHA binding sites as a result of a decrease in association rate of the ligand to the receptors. At the same time the affinity for NE was slightly altered too. Moreover the effect of guanyl nucleotides on agonist binding was found

to be decreased in ischemia as compared to their respective sham-operated controls ["Ki" shift ("-fold") was 2.1 and 3.81, respectively]. Similar changes were seen in the tissue obtained 72 hours but not 24 hours after the carotid artery clip release.

Significance for Biomedical Research and the Program of the Institute:  
The investigations of nonvascular and vascular cerebral receptors and their interrelationship with the changing levels of neurotransmitters are of utmost importance in cerebral ischemia and ischemic edema since their altered function has been implicated to play a role in aggravating this disease process.

Proposed Course of the Project: Part of this study was presented at the American Society for Neurochemistry meeting in Richmond, Virginia, March 1981.

Publications: None



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02463-01 LNNS
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less) The effect of central nervous tissue on cerebral endothelial properties		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: M. Spatz Head, Section on Neurocytobiology LNNS NINCDS		
COOPERATING UNITS (if any) Dr. Ronald F. Dodson, Division of Experimental Pathology, East Tyler Chest Hospital, Tyler, Texas		
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences		
SECTION Section on Neurocytobiology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.4	PROFESSIONAL: 0.1	OTHER: 0.3
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The preliminary investigations have shown a stimulation of cerebral endothelial cell line growth co-cultured with cerebellar explants.		

## Project Description:

**Objectives:** The cultivated endothelial cells derived from cerebral microvessels of the rat (Spatz et al., Brain Res. 191, 577-588, 1980), in mouse (DeBault et al., In Vitro 15, 473-487, 1979) possess similar characteristic features in regard to the blood-brain barrier (BBB) as those observed in either in vivo or in vitro studies of the BBB. However, gap but not tight junctions, which are one of the main characteristics of BBB, developed in these cultures. Ontogenetically the tight junctions and other vascular BBB features appear only after the extracerebral vessels penetrate the brain in animals and human. Therefore, we undertook to investigate the overall influence of the nervous tissue on the endothelial properties.

**Methods Employed:** The organotypic cultures of cerebellum and cerebral endothelial cells were established separately. Then aliquots of the endothelial cells (third generation) have been cocultured with a 3-week-old organotypic cerebellar culture in a Maximow's assembly system for 2-3 weeks. The nutrient medium for the establishment and propagation of each culture was the same as that described for the endothelium by Spatz et al. (Brain Res. 191, 577-582, 1980). The cultures were washed and fed biweekly. At the end of the experimental period the activities of alkaline phosphatase, butyrylcholinesterase and  $\gamma$ -glutamyl transpeptidase as well as the cellular incorporation of  $^3\text{H}$  thymidine were evaluated histochemically and by radioautography, respectively.

**Major Findings:** This project is still in the preliminary stages of investigation. However, so far, the following observations have been made concerning the effects of nervous tissue on the endothelium in culture. 1) An enhancement in the activities of alkaline phosphatase,  $\gamma$ -glutamyl transferase but not of butyryl cholinesterase was seen in the cerebral endothelial cells cocultured with cerebellar explants. 2) The cellular incorporation of  $^3\text{H}$  thymidine into the endothelium was greater in the cells cocultured with the cerebellar than in those cultured alone. The observed increased reactivity of the endothelial cells was most marked in regions in which the endothelial cells were close to the cellular outgrowth of the cerebellar explants.

**Significance for Biomedical Research and the Program of the Institute:** The study of endothelial cells derived from cerebral microvessels alone or in the presence of other cellular elements of nervous tissue presents the investigation and characterization of the endothelial function in the living state without or under the influence of other cell types. This approach will add another dimension to the study of cerebral endothelium processes related to the BBB permeability under normal and pathological conditions.

**Proposed Course of the Project:** These studies will focus on biochemical immunocytochemical and electron microscopical evaluation of growth pattern and junctional formation of the endothelium in cultures.

**Publications:** None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  <div style="text-align: center;">Z01 NS 01995-09 LNNS</div>																								
PERIOD COVERED October 1, 1980 to September 30, 1981																										
TITLE OF PROJECT (80 characters or less)  Morphological studies of myelin formation, breakdown and regeneration																										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																										
<table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI:</td> <td style="width: 30%;">H. deF. Webster</td> <td style="width: 30%;">Associate Chief</td> <td style="width: 10%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>B.D. Trapp</td> <td>Staff Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>H. Shii</td> <td>Visiting Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>F. Omlin</td> <td>Guest Worker</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>J. Martin</td> <td>Senior Staff Fellow</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>R.H. Quarles</td> <td>Chief, Myelin and Brain Development Section</td> <td>DMNB NINCDS</td> </tr> </table>			PI:	H. deF. Webster	Associate Chief	LNNS NINCDS	Other:	B.D. Trapp	Staff Fellow	LNNS NINCDS		H. Shii	Visiting Fellow	LNNS NINCDS		F. Omlin	Guest Worker	LNNS NINCDS		J. Martin	Senior Staff Fellow	LNNS NINCDS		R.H. Quarles	Chief, Myelin and Brain Development Section	DMNB NINCDS
PI:	H. deF. Webster	Associate Chief	LNNS NINCDS																							
Other:	B.D. Trapp	Staff Fellow	LNNS NINCDS																							
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	F. Omlin	Guest Worker	LNNS NINCDS																							
	J. Martin	Senior Staff Fellow	LNNS NINCDS																							
	R.H. Quarles	Chief, Myelin and Brain Development Section	DMNB NINCDS																							
COOPERATING UNITS (if any) Developmental and Metabolic Neurology Branch, NINCDS; Department of Neurology and Neuropathology, Massachusetts General Hospital and Harvard Medical School, Boston, Mass.																										
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences																										
SECTION Section on Cellular Neuropathology																										
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																										
TOTAL MANYEARS: <div style="text-align: center;">5.4</div>	PROFESSIONAL: <div style="text-align: center;">3.6</div>	OTHER: <div style="text-align: center;">1.8</div>																								
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;"> <input type="checkbox"/> (a) HUMAN SUBJECTS         </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (b) HUMAN TISSUES         </div> <div style="width: 30%;"> <input type="checkbox"/> (c) NEITHER         </div> </div> <div style="display: flex; justify-content: space-between; align-items: flex-start; margin-top: 5px;"> <div style="width: 30%;"> <input type="checkbox"/> (a1) MINORS         </div> <div style="width: 30%;"> <input type="checkbox"/> (a2) INTERVIEWS         </div> </div>																										
SUMMARY OF WORK (200 words or less - underline keywords)  The long range goal of this project is to combine <u>immunocytochemical methods</u> with <u>light and electron microscopy</u> to study cellular mechanisms of <u>myelin</u> <u>formation, breakdown and regeneration</u> . Nervous tissues from experimental animals and patients with demyelinating diseases have been studied in the following current projects: 1) Distribution of myelin-associated glycoprotein (MAG) and basic protein in acute stages of <u>CNS Wallerian degeneration</u> 2) Distribution of MAG and proteins in compact peripheral myelin (P <sub>0</sub> , P <sub>1</sub> , and P <sub>2</sub> ) in lesions of <u>idiopathic polyneuritis</u> 3) <u>Localization of P<sub>0</sub> myelin protein</u> in Schwann cells and myelin during <u>PNS myelination</u> .																										

## Project Description:

**Objectives:** To study the distribution of MAG and BP in rat optic nerves during early stages of Wallerian degeneration. 2) To study the distribution of MAG and compact peripheral myelin proteins  $P_0$ ,  $P_1$ , and  $P_2$  in lesions of idiopathic polyneuritis. 3) To localize  $P_0$  protein in Schwann cells during peripheral myelination.

**Methods Employed:** 1) CNS Wallerian degeneration was produced by ocular enucleation in young adult Osborne Mendell rats. One, two, and four days after enucleation, nerves were removed and immunostained with MAG and BP antisera according to the PAP methods. At these intervals, other nerves were processed for electron microscopic study. 2) Sections of lesions from 17 cases of idiopathic polyneuritis were immunostained with antisera to MAG and to three compact myelin proteins,  $P_0$ ,  $P_1$ , and  $P_2$  according to the peroxidase-antiperoxidase (PAP) method. 3) Trigeminal and sciatic nerves from newborn, 7 day, and adult rats were immunostained with antiserum to  $P_0$  according to the PAP method. Serially cut adjacent thin sections were examined and electron micrographs were taken of Schwann cells that were immunostained in the adjacent one micrometer sections. To localize  $P_0$  in Schwann cell cytoplasm and myelin membranes, the distribution of immunostaining was mapped on electron micrographs of identical areas found in the adjacent thin sections.

**Major Findings:** 1) Early changes were identified in some myelinated fibers 48 hours after enucleation. The staining pattern of periaxonal MAG was disrupted and many fragments were very densely stained. Abnormal BP staining was also observed in myelin sheaths surrounding these axons. After 4 days, almost all periaxonal regions were affected and more advanced degeneration was found in myelin sheaths. 2) In early idiopathic polyneuritis lesions, there were focal abnormalities in  $P_2$ ,  $P_0$ , and  $P_1$  (similar if not identical to BP in the CNS) immunostaining of paranodal and intranodal myelin. No single protein was affected selectively and lesions occurred in fibers of all sizes, not just in larger fibers selectively stained by  $P_2$  antiserum. Early changes in MAG immunostaining occurred only in regions where myelin immunostaining also was abnormal. More severe, later changes in the distribution of  $P_0$ ,  $P_2$ , BP, and MAG were consistent with the sequence of myelinated fiber alterations seen in segmental demyelination and Wallerian degeneration. In regenerating fibers, MAG antiserum stained periaxonal regions intensely; thin, regenerating myelin sheaths were stained by  $P_0$  and  $P_1$  antiserum but not by  $P_2$  antiserum. 3) The first  $P_0$  staining was found around axons and/or in Schwann cells that had established a 1:1 relationship with axons. Myelin sheaths with as few as 3 lamellae were immunostained and could be detected by light microscopy. Very thin sheaths often stained less intensely and part of their circumference frequently was unstained. As myelination progressed, intensely stained myelin rings became much more numerous and in adult nerves, all sheaths were intensely and uniformly stained. Particulate  $P_0$  staining also was observed in juxtanuclear areas of Schwann cell cytoplasm. It was most prominent during development, then decreased, but still was detected in adult nerves. The cytoplasmic areas stained by  $P_0$  antiserum were rich in Golgi membranes.



Significance to Biomedical Research and the Program of the Institute:

1) The results showed that the first changes detected in Wallerian degeneration occurred in axonal organelles and the axon membrane. These preceded abnormalities in the distribution of MAG and BP. 2) Since we found MAG changes only in areas where the distribution of myelin proteins was abnormal, we suggest that myelin is the primary target in this demyelinative process. Direct interaction of inflammatory cells and myelin probably initiates myelin breakdown with associated secondary changes in Schwann cells. 3) Our immunostaining method for studying the distribution of  $P_0$  in epon sections is sensitive, specific, and the results add significantly to our understanding of where  $P_0$  occurs in Schwann cells as they form myelin.

Proposed Course of the Project: To be continued. The findings were presented at meetings of the American Association of Neuropathologists, the first meeting of the European Society of Neuropathologists, and the Peripheral Nerve Study Group.

Publications:

Webster, H. deF. and Sternberger, N. H.: Morphological features of myelin formation. In Baumann, N. (Ed.): Neurological Mutants Affecting Myelination: Research Tool in Neurobiology. INSERM Symposium No. 14. Amsterdam, Elsevier/North-Holland, 1980, pp. 73-86.

Cullen, M. J., DeVries, G. H., Webster, H. deF.: Freeze-fracture characterization of isolated myelin and axolemma membrane fractions. Brain Res. 1981 (in press).

Matthieu, J.-M., Mottet, S., Kraus-Ruppert, R., Cohen, S. R., and Webster, H. deF.: Effects of Colchicine on myelination of rabbit optic nerve: A biochemical study. Neurotoxicology, 1981 (in press).

Webster, H. deF., Trapp, B. D., Sternberger, N. H., and Quarles, R. H.: Myelin forming glial cells: Morphological and immunocytochemical observations. In Garrod, D. R. & Feldman, J. D. (Eds.): Symposium on Development in the Nervous System. Cambridge, Cambridge University Press, 1981, pp. 265-288.

Trapp, B. D., Itoyama, Y., Sternberger, N. H., Quarles, R. H., and Webster, H. deF.: Immunocytochemical localization of  $P_0$  protein in Golgi membranes and myelin of developing rat Schwann cells. J. Cell Biol. 90:1-6, 1981.

Trapp, B. D., Marangos, P. G., and Webster, H. deF.: Immunocytochemical localization and developmental profile of neuron specific enolase (NSE) and non-neuronal enolase (NNE) in aggregating cell cultures of fetal rat brain. Brain Res. 1981 (in press).

Schober, R., Itoyama, Y., Sternberger, N. H., Trapp, B. D., Richardson, E. P., Asbury, A. K., Quarles, R. H., and Webster, H. deF.: Immunocytochemical study of  $P_0$  glycoprotein,  $P_1$  and  $P_2$  basic proteins, and myelin-associated glycoprotein (MAG) in lesions of idiopathic polyneuritis. J. Neuropath. & Applied Neurobiol. 1981 (in press).



Publications:

Itoyama, Y., Webster, H. deF., Sternberger, N. H., Richardson, E. P., Walker, D. L., Quarles, R. H., and Padgett, B. L.: Distribution of papovavirus, myelin-associated glycoprotein and myelin basic protein in progressive multifocal leukoencephalopathy lesions. Annals of Neurology, 1981 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 01442-15 LNNS
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less) Permeability of Cellular Layers in the Vertebrate Nervous System		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: T. S. Reese Head, Section on Functional Neuroanatomy LNNS NINCDS Other: B. Kachar Visiting Fellow LNNS NINCDS		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences		
SECTION Section on Functional Neuroanatomy		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">0.6</div>	PROFESSIONAL: <div style="text-align: center;">0.5</div>	OTHER: <div style="text-align: center;">0.1</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;"> <input type="checkbox"/> (a) HUMAN SUBJECTS   <input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS         </div> <div style="width: 30%;"> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) The substructure of tight junctions is under investigation by <u>direct freezing</u> techniques that avoid any chemical fixation, and serve to increase the resolution of its individual components. The tight junction is made of rod-shaped structures embedded in the central lipophilic domain of each of its paired component membranes. This view revises the earlier view that the tight junction is a row of intramembrane particles in register with a similar row in an adjacent membrane. These results are expected to lead to an understanding of how tight junctions serve in the blood-brain barrier system to prevent small charged solutes from entering the brain.		

## Project Description:

Objectives: To understand at the molecular level the structure of tight junctions pertinent to their function as intercellular barriers.

Methods Employed: Epithelia from various organs are chosen for examination on the basis of the accessibility of their tight junctions to rapid-freezing and the number of well-frozen tight junctions available in freeze-fracture replicas. The junctions at blood-brain barriers serve neither of these purposes well, but their structure differs little from those in a variety of epithelia outside the brain, which is why the blood-brain barrier is not studied directly at this stage. Epithelia cells which have been rapid frozen at close to ideal in vivo conditions are replicated directly after freeze-fracture, or after partial dehydration (freeze-etching) to reveal the relationships of the cytoskeleton to the tight junctions. Some replicas are made at very high levels of resolution with methods explored in Project No. Z01 NS 01881-11. These replicas are then subjected to stereomicroscopy.

Major Findings: These experiments show, for the first time, the structure of tight junctions which have not been exposed to fixatives or other chemical treatments in well-frozen areas; the basic junctional element is a continuous bar embedded in the lipophilic center of the membrane. This contrasts with the view of the tight junction as a row of intramembrane particles, which is often the view obtained with conventional preparative methods. Views showing both membranes participating in a tight junction show that each contains a bar which contacts the adjacent bar, not necessarily along an axis perpendicular to the membrane plane.

Significance to Biomedical Research and the Program of the Institute: Tight junctions are known to impede intercellular movement of any solute which does not readily cross the cell membrane. An exact understanding of how a contact between cells can acquire the properties of the cell membrane itself depends on defining the arrangement of lipidic membrane components at the tight junction; our direct freezing and high resolution freeze-fracture methods appear to provide a means to do this. Then it may be possible to define how a variety of drugs and conditions may disrupt the tight junctions which are the basis of the blood-brain barrier.

Proposed Course of the Project: This work was begun in the middle of this year with the arrival of Bechara Kachar, and is quite preliminary at this stage. We plan to continue with the observations as outlined above, and in particular with study of junctions freeze-fractured at very low temperatures to avoid possible lipidic rearrangements during and after freeze-fracturing.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 01881-11 LNNS
PERIOD COVERED      October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less) Structural basis of synaptic transmission		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:   Other:	T. S. Reese  G. Benshalom C. P. Ko K. J. Lynch R. L. Ornberg D. W. Pumplin V. Verma J. Walrond	Head, Section on Functional Neuroanatomy Visiting Fellow Guest Worker Guest Worker Staff Fellow Guest Worker Visiting Fellow Guest Worker  LNNS NINCDS LNNS NINCDS LNNS NINCDS LNNS NINCDS LNNS NINCDS LNNS NINCDS LNNS NINCDS LNNS NINCDS
COOPERATING UNITS (if any) J.E. Heuser, Dept. of Physiology & Biophysics, Wash. Univ. St. Louis, MO. S. Nakajima, Purdue University, West Lafayette, IN		
LAB/BRANCH  Laboratory of Neuropathology and Neuroanatomical Sciences		
SECTION  Section on Functional Neuroanatomy		
INSTITUTE AND LOCATION  NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:  7.5	PROFESSIONAL:  5.0	OTHER:  2.5
CHECK APPROPRIATE BOX(ES)  <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) This project seeks to determine the location and mechanism of <u>neurotransmitter</u> secretion and reception. Rapid freezing and subsequent <u>freeze-fracture</u> of <u>synapses</u> expose fleeting structural changes in the cell membrane accompanying <u>discharge of synaptic vesicles</u> . By these means, the prodromata and aftermath of synaptic vesicle exocytosis have been determined. This approach has been extended to other secretory cells where details surrounding the initiation of secretion can be better understood. New methods have been developed to use rapid freezing to localize <u>calcium</u> in neural tissue in different states of activity in order to define its role in controlling these states. These methods have succeeded in defining a special intracellular membrane system which stores calcium. This work is significant in that it defines the normal structure of synapses and relates normal variations in structure to different functional states. Thus, it becomes possible to distinguish pathological changes in structure. The current program also includes freeze-fracture of <u>developing</u> and <u>degenerating synapses</u> which will aid in understanding of both normal development and <u>development failures</u> in the brain and peripheral nervous system.		

## Project Description:

Objectives: Synapses are sites where electrical signals pass between neurons or between neurons and muscle cells. This project seeks to establish the structural basis and mechanism of synaptic transmission in the central and peripheral nervous systems in both adult and immature animals, and in tissue cultured neurons and muscles.

Methods Employed: Tissues are prepared for freeze-fracturing or for freeze-substitution by a new technique which rapidly freezes tissue surfaces in one msec. Thus, tissue prepared for freeze-fracturing experiences no chemical treatment, while tissue prepared for sectioning is fixed at low temperatures in non-aqueous solvents. Our main purpose is to visualize the events which accompany and immediately follow transmitter secretion. Earlier experiments consisted of giving a nerve-muscle preparation a single shock and freezing it from 3 to 1000 msec later in preparation for either freeze-fracturing or freeze-substitution. The initial stages in secretion are studied in a similar manner in *Limulus* amoebocytes (blood cells), a preparation chosen because the secretory granules are very large and the secretion proceeds precipitously after these cells contact endotoxin. Growth cones from cultured sympathetic ganglion cells (rat) are also being studied by freeze-substitution.

In order to localize calcium in muscle and neural tissues, they are rapidly frozen and then freeze substituted in tetrahydrofuran (THF). This method was developed by measuring the loss of  $^{45}\text{Ca}^{++}$  from tissues during preparation to minimize loss, and by localizing calcium in frog muscles where its natural distribution is already known; the introduction of THF for this purpose has greatly reduced diffusion of calcium. Calcium is ultimately localized in tissues with an electron microprobe equipped with a cold stage and an energy-loss spectrometer.

In the last year we have introduced a method for fracturing tissues at very low temperatures ( $\sim 20^\circ\text{K}$ ) and shadowing them with iridium-tantalum alloy to yield very high levels of resolution with little distortion from the fracturing process. Tissues prepared in this way are then etched (a modification of the freeze-drying process) before shadowing in order to uncover details of cytoplasmic structure.

Other studies of synapses still depend on conventional freeze-fracture techniques using chemical fixatives. The giant synapse from the squid has been examined because its physiological condition can be defined so precisely. Squid axons are then put in an aldehyde fixative, frozen, freeze-fractured, and the resulting replicas of split membranes examined at high resolution in an electron microscope. A similar approach is also being used to study differences between synapses at fast and slow muscles in the frog, and the responses of these nerves and muscles to nerve resection. Another project has been initiated to examine the membrane ultrastructure of temperature sensitive neurological mutants affecting neuromuscular junctions in *Drosophila*.

Major Findings: By freeze-fracturing rapid frozen neuromuscular synapses, it has been possible to determine the fate of synaptic vesicle membrane after synaptic



vesicles fuse with the presynaptic plasmalemma. In less than 0.1 sec, the vesicle membrane is completely flattened out into the plasmalemma. Components of the vesicle membrane, appearing as particles after freeze-fracturing, then spread out to be collected a second later into particle islands which are parts of the coated vesicle system. The final fate of these components of the vesicle membrane is to be reincorporated into synaptic vesicles. This finding of particle recycling extends earlier work of the section showing that local recycling of synaptic vesicles replaces those lost during synaptic activity.

The initial stages in membrane interaction which lead to membrane fusion and exocytosis are so rapid at the frog neuromuscular junction that we turned to a preparation in which we could examine much larger secretory granules, and where we expected the initial stages of secretion to be more long-lived. *Limulus* amebocytes secrete precipitously within seconds after exposure to endotoxin, so the initiation of this process can be studied by freezing at different short intervals after application of endotoxin. The first change is a small perforation in the plasmalemma which rapidly widens, suggesting that exocytosis begins at a point rather than along a wide front of intermembrane contact.

The rapid freezing technique is also applicable to localizing calcium in tissues, provided the frozen tissue is subsequently freeze-substituted in the presence of oxalic acid. Recently we have introduced tetrahydrofuran for this processing which results in a large decrease in the diffusion of that calcium which is not associated with intracellular organelles. In muscle treated in this manner, we could detect no washout of calcium, and the calcium was localized with an electron probe at its expected positions in terminal cisterns of sarcoplasmic reticulum. We have applied this approach to stimulated synapses, where we found that the calcium which enters from the outside is sequestered in endoplasmic reticulum. We have also identified a similar system in amebocytes which sequesters the intracellular calcium after it has initiated the secretory events.

The method for high resolution freeze-etch preparation of untreated tissue that we introduced this year has been applied to the post synaptic active zone in auditory brain-stem synapses. It has revealed a system of 4nm active zone related filaments which contact post synaptic intramembrane structures believed to be receptors. These filaments are contacted on their other ends by a subsynaptic meshwork of microfilaments. These structural specializations at the synapse may stabilize receptors there, and confer a stable shape on this region of the cell membrane.

Comparative studies of other types of synapses were made using either rapid-freezing or conventional fixation to prepare them for freeze-fracturing. The giant synapse in the squid was shown to have well-defined synaptic vesicle release areas. We have serial-sectioned whole squid synapses to determine the numbers of membrane particles associated with release areas ( $1.1 \times 10^4$ ) and shown that this number yields a reasonable single particle conductance when each particle is assumed to be a single calcium channel, which substantiates our earlier suggestions that these particles are, in fact, calcium channels.

The extent of local recycling at the frog neuromuscular junction was measured by stimulating isolated synapses for up to 48 hours and then evaluating depletion of synaptic vesicles and other membranes. No depletion of synaptic vesicles has

been found, even in preparations where axoplasmic supplies of membrane were blocked, either by initiating axoplasmic transport with colchicine or by ligating the nerve near the muscle. This preparation is now being studied with rapid-freezing and freeze substitution after stimulation in ferritin in order to see how new synaptic vesicles are formed once synaptic membranes are recovered.

The responses of nerve terminals to nerve resection are also being studied with conventional freeze-fracture techniques. The results suggest that at the moment when evoked transmitter release fails, the synaptic active zone particles disperse, and that spontaneous release is blocked only when the nerve is engulfed by Schwann cells, several hours later.

Significance to Biomedical Research and the Program of the Institute: One of the most immediately practical aspects of these studies on synapses is that they define the normal structure of various types of synapses in a variety of functional states. This knowledge will permit distinction between normal and pathological, as well as between resting and active synapses, with the electron microscope. In diseases involving peripheral nerve-muscle synapses, it becomes possible to distinguish pathological states from changes resulting from increased or decreased activity. Our new studies on the post-synaptic active zone and on development and degeneration at synapses may reveal, on a cellular level, why development or repair of synaptic systems is sometimes unsuccessful. Finally, our new directions in understanding how cells handle calcium will make it possible to study how these systems interact with the wide variety of drugs and diseases which affect our nervous system.

Our program of developing and adapting the rapid freezing and freeze-fracture technique to study neural structure has been helpful to other program areas of NINCDS, as evidenced by the fact that major programs in neuroviruses, otolaryngology, and multiple sclerosis have found it important to make, with our assistance, major commitments to setting up facilities to perform research with this technique. In every instance, their primary investigators were trained in this technique in the Section on Functional Neuroanatomy.

Proposed Course of the Project: Much of the work outlined above is currently being prepared for publication, or has been submitted. The final work on rapid changes in frog neuromuscular synapses which accompany transmitter release has been published. The work on the initial step in secretion in amebocytes is also published.

A major new direction is to extend the rapid freezing and freeze-substitution techniques to new areas of synaptic and membrane physiology. In particular, we will take advantage of a new method we have developed to localize calcium to see how it is stored and released in a variety of neural tissues. We expect that the analytical work on calcium distribution will be greatly aided by our new high resolution analytical scanning transmission electron microscope which is now functional. A second direction is to use the freeze-fracture technique to study developing and degenerating synapses. A third direction is to use the rapid freezing in conjunction with high resolution freeze-fracture technique to look at the substructure of neuronal cytoplasm and, perhaps, to define configurational changes in intramembrane proteins during neural activity.

Publications:

Smith, J. E., and Reese, T. S.: Use of aldehyde fixative to determine the rate of synaptic transmitter release. J. Exp. Biol. 89: 19-29, 1980.

Ornberg, R. L., and Reese, T. S.: A freeze-substitution method for localizing divalent cations: examples from secretory systems. Fed. Proc. 39: 2802-2805, 1980.

Rees, R. P., and Reese, T. S.: New structural features of freeze-substituted neuritic growth cones. Neuroscience 6: 247-254, 1981.

Heuser, J. E., and Reese, T. S.: Structural changes following transmitter release at the frog neuromuscular junctions. J. Cell Biol. 88: 564-580, 1981.

Ornberg, R. L., and Reese, T. S.: Beginning of exocytosis captured by rapid freezing of Limulus amebocytes. J. Cell Biol. 90: 40-54, 1981.

Gulley, R. L., and Reese, T. S.: Cytoskeletal organization at the post synaptic complex. J. Cell Biol. (in press).

Ko, C.-P.: Electrophysiological and freeze fracture studies of changes following denervation at frog neuromuscular junctions. J. Physiol. (in press).

Pumplin, D. W., Resse, T. S., and Llinas, R.: Are presynaptic active zone particles calcium channels? Proc. Natl Acad. Sci. (in press).





Project Description:

Objectives: To see whether assemblies of particles in astrocyte cell membranes are associated with cytoplasmic proteins and assembly alterations in cold lesions.

Methods Employed: Astrocyte cultures are exposed to agents that act on cytoplasmic proteins: cytochalasin B and D, which disassociate actin filaments, and colchicine and vinblastine which disaggregate microtubules. Injury to the cerebral cortex, including the glia limitans, was produced by placing a 1 mm-wide brass rod, at dry-ice temperature, on the skull of 9-day-old rats for 2 seconds. The tissues and cultures were fixed and freeze-cleaved.

Major Findings: Distribution of assemblies is differentially altered by agents affecting cytoskeleton. Microfilament dissociation results in clumping of assemblies, microtubule disruption in "cap"-like aggregation. At the site of cold lesion, assemblies are tightly aggregated very much like those after denaturation with guanidine; at lesion periphery, the number of assemblies are greater than intact areas.

Significance: The differential rearrangement of assemblies strongly suggests that they are physically connected with the cytoskeleton. Focal freezing causes changes closely akin to protein denaturation and to other types of gliosis.

Proposed Course: To alter, with physiological agents, the cytoplasmic and extracellular matrix to see if configurational changes occur. To isolate the protein of the assemblies by separation of astrocyte membrane for SDS-gel electrophoresis and to further probe the changes during gliosis.

Publications: Brightman, M. W., Anders, J. J., and Rosenstein, J. J.: Specializations of nonneuronal cell membranes in the vertebrate nervous system. In Federoff, S. (Ed.): Advances in Cellular Neurobiology, Vol. 1. New York, Academic Press, 1980, pp. 3-29.

Dorovini-Zis, K., Anders, J. J., and Brightman, M. W.: Cerebral endothelium, blood-brain barrier and the astrocyte membrane. In Cunha-Vas, J. G. (Ed.): The Blood-Retinal Barriers. New York, Plenum Press, 1980, pp. 65-79.

Anders, J. J. and Brightman, M. W.: Orthogonal assemblies of intramembranous particles - An attribute of the astrocyte. In Acosta Vidrio, E. and Federoff, S. (Eds.): Progress in Clinical and Biological Research, Vol. 59A. Eleventh International Congress of Anatomy, Part A: Glial and Neuronal Cell Biology. New York, Liss, Inc., 1981, pp. 21-35.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02086-08 LNNS																
PERIOD COVERED      October 1, 1980 to September 30, 1981																		
TITLE OF PROJECT (80 characters or less)  Regeneration in Peripheral and Central Nerves																		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">T. Kirino</td> <td style="width: 35%;">Visiting Fellow</td> <td style="width: 15%;">LNNS NINCDS</td> </tr> <tr> <td>Other:</td> <td>D. Rubin</td> <td>Guest Worker</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>J. Rosenstein</td> <td>Guest Worker</td> <td>LNNS NINCDS</td> </tr> <tr> <td></td> <td>M. W. Brightman</td> <td>Head, Section on Neurocytology</td> <td>LNNS NINCDS</td> </tr> </table>			PI:	T. Kirino	Visiting Fellow	LNNS NINCDS	Other:	D. Rubin	Guest Worker	LNNS NINCDS		J. Rosenstein	Guest Worker	LNNS NINCDS		M. W. Brightman	Head, Section on Neurocytology	LNNS NINCDS
PI:	T. Kirino	Visiting Fellow	LNNS NINCDS															
Other:	D. Rubin	Guest Worker	LNNS NINCDS															
	J. Rosenstein	Guest Worker	LNNS NINCDS															
	M. W. Brightman	Head, Section on Neurocytology	LNNS NINCDS															
COOPERATING UNITS (if any)  None																		
LAB/BRANCH      Laboratory of Neuropathology and Neuroanatomical Sciences																		
SECTION      Section on Neurocytology																		
INSTITUTE AND LOCATION      NINCDS, NIH, Bethesda, Maryland 20205																		
TOTAL MANYEARS: 3.1	PROFESSIONAL: 2.8	OTHER: .3																
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																		
SUMMARY OF WORK (200 words or less - underline keywords) The <u>transplantation</u> of autonomic ganglia has been extended to CNS tissue. Hypothalamic tissue from 17-day-old rat fetuses has been transplanted to the intact surface over the <u>area postrema</u> of 6 day old rats. Three months later, the hypothalamic graft contains many neurons and a rich neuropil including what appear to be neurosecretory axons. Survival of these large transplants is due to establishment of anastomoses with meningeal blood vessels. During degeneration of hypoglossal (XIIn) axons after nerve crush, the neuron-specific enolase (NSE) of the XIIn neurons begins to fall during the first 10 days and returns to normal levels between 30 and 45 days. There does not appear to be a rise in non-neuronal enolase (NNE) during the fall in NSE; there is no switch from one type of enolase to the other as we have found during development. This first demonstration of a change in the amount of glycolytic enzyme during degeneration and regeneration has also revealed small neurons that are unaffected by nerve crush. They appear to be interneurons.																		

Project Description:

Objectives: To see whether there is specificity between regenerated neuro-hemal contacts and whether a neuron-specific glycolytic enzyme changes during regeneration.

Methods: Blocks of hypothalamus from 17-day-old rat fetuses are transplanted to the intact surface of the area postrema and the brain fixed weeks to months later. The hypoglossal nerve is crushed in rats and the levels of NSE in its nucleus monitored immunocytochemically during degeneration and regeneration.

Major Findings: Large masses of fetal brain tissue, including what may be neurosecretory neurons, can survive for months after transplantation to intact brain surfaces. Changes in levels of a neuron-specific glycolytic enzyme accompany neuronal degeneration and regeneration.

Significance: Functional neuroendocrine transplants without trauma to host cerebral tissue is now a likelihood. Changes in levels of a neuron-specific glycolytic enzyme may reflect altered metabolic needs during degeneration and suggest that the degenerating motor neuron does not undergo "de-differentiation".

Proposed Course: To determine whether the transplants include regenerating neurosecretory axons containing vasopressin that can alleviate diabetes insipidus and whether the recovery of neuron-specific enzyme depends on re-innervation of target cells.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02144-07 LNNS
PERIOD COVERED      October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less) The Blood-Brain Barrier. Bypassed With Ganglion Implants. Former Title: Effects of Hypertension on the Permeability of Cerebral Endothelium to Proteins		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:                    M. W. Brightman Other:                J. Rosenstein	Head, Section on Neurocytology Guest Worker	LNNS NINCDS LNNS NINCDS
COOPERATING UNITS (if any)		
LAB/BRANCH      Laboratory of Neuropathology and Neuroanatomical Sciences		
SECTION            Section on Neurocytology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:      1.3	PROFESSIONAL:          .9	OTHER:                  .4
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) When <u>macromolecules</u> , such as horseradish peroxidase (HRP), are injected into the blood, they are prevented from entering <u>CSF</u> and <u>extracellular fluid</u> of the brain by the blood-brain and blood-CSF barriers: the tight junctions of cerebral endothelium and choroid plexus epithelium account, respectively, for these exclusions. Both barriers are circumvented by transplanting pieces of superior cervical ganglia (SCG) to the intact <u>surface</u> of the brain because (i) the <u>capillaries</u> of ganglia are fenestrated and <u>permeable</u> to protein, (ii) the <u>extra-cellular clefts</u> of the SCG become confluent with those of brain and (iii) there are no belts of tight junctions between normal or reactive astrocytes of the subpial glia to prevent the extracellular <u>penetration</u> of macromolecules from blood into brain. The extracellular compartment of a peripheral ganglion transplant becomes perfectly confluent with that of the brain and is thus able to transfer blood-borne molecules into brain.		

Project Description:

Objectives: To see whether a transplanted, peripheral ganglion affects the blood-brain barrier to protein.

Methods: Pieces of superior cervical ganglion are transplanted to the intact pial surface of the medulla and cerebellum and, after weeks to months, horseradish peroxidase (HRP) is injected into the femoral vein. From 10 to 30 minutes later, the brains are fixed.

Major Findings: Within 10 minutes, HRP leaves the permeable vessels of the SCG grafts to enter the extracellular clefts of the graft and thence those of the underlying brain. An important interaction between graft and host-tissue is establishment of continuity between the extracellular compartment.

Significance: A consequence of transplantation to undamaged surfaces of the brain is a melding of the two tissues so that their extracellular spaces become continuous. Since these brain surfaces are accessible without damage, transplantation of peripheral ganglia could be a means of continuously infusing blood-borne agents into the brain from the periphery.

Proposed Course: To determine how rapidly this entry is and to extend the findings to biogenic amines that are electron dense.

Publications: Rosenstein, J. M. and Brightman, M. W.: Arrest and migration of cerebellar neurons towards grafts of autonomic ganglion. Peptides 1 (Suppl. 1): 221-227, 1980.

Rosenstein, J. M. and Brightman, M. W.: Anomalous migration of central nervous tissue to transplanted autonomic ganglia. J. Neurocytol. 10: 1981 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02286-05 LNNS
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less) Mechanism of cerebral hemorrhages		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: J. Cammermeyer                      Guest Worker                      LNNS NINCDS		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences		
SECTION Office of the Chief, LNNS		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.3	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  Petechial cerebral hemorrhages induced by oil embolism in material fixed by perfusion are compared with those in material fixed by immersion.		



Project Description:

Objectives: To assess morphologic distinctions between petechial hemorrhages in material fixed by immersion and those fixed by perfusion.

Methods Employed: Injection of fat in systemic circulation of cats. Fixation by perfusion or by immersion after varying post-injection intervals. Intracardial injection of India ink during the perfusion. Embedding in paraffin or plastic. Histologic techniques for staining of erythrocytes and vascular walls.

Major Findings: Petechial hemorrhages and larger hemorrhagic infarctions composed of fresh erythrocytes aggregated near sites of vascular ruptures. They are of different appearance after use of the two types of fixation.

Significance to Biomedical Research and the Program of the Institute: An assessment of the factors contributing to hemorrhages may help to determine whether these hemorrhages occur during life or whether they can be the cause of death. Formulation of therapeutic measures as well as interpretation of hemorrhages as the cause of death will be dictated by the results of morphologic studies. The question is whether interpretation based on experimental material fixed by perfusion can be applied to material fixed by immersion, as is the case of human material.

Proposed Course of the Project: To supplement the immersion fixed material with longer post-operative intervals.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02362-03 LNNS
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less) Effect of dimethyl sulfoxide on the histochemical demonstration of glycogen in the perfusion fixed brain		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <div style="display: flex; justify-content: space-between;"> <span>PI: J. Cammermeyer</span> <span>Guest Worker</span> <span>LNNS NINCDS</span> </div>		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences		
SECTION Office of the Chief, LNNS		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">0.7</div>	PROFESSIONAL: <div style="text-align: center;">0.7</div>	OTHER: <div style="text-align: center;">0</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS   <input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS         </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) <p>When normal Netherlands dwarf rabbits were perfused with dimethyl sulfoxide (DMSO)-containing solutions, the brains exhibited pericapillary foci with acute tissue destruction and perivenous areas in which <u>neurons</u> were filled with glycogen. Glycogen was also discernible in <u>microglial cells</u> and <u>oligodendrocytes</u>. Because of the irregular distribution of glycogen-filled cells this method of fixation is not recommended for systematic studies on the distribution of glycogen in normal and experimental animals. This project has been completed and the manuscript is being prepared for publication.</p>		

Project Description:

Objectives: To prevent polarization of glycogen in Purkinje cells by adding a drug to the fixative which will enhance infiltration of tissues.

Methods Employed: Glycogen was stained by the dimedone periodic acid-Schiff technique in paraffin sections from brains fixed by perfusion with a modified Bouin's solution mixed with dimethyl sulfoxide in varying concentrations.

Major Findings: Irregular perivenous areas contain neurons filled with glycogen.

Purkinje cells do not display polarization of glycogen.

Glycogen is demonstrated in cerebellar granule cells, oligodendrocytes and microglial cells as well as in astrocytes.

Significance to Biomedical Research and the Program of the Institute: The adoption of a special fixative in which DMSO is added made it possible to demonstrate glycogen to a degree not previously seen except in extraordinary conditions of hibernation, recovery from narcosis, X-irradiation and seasonal variations. These observations were typical of the Netherlands dwarf rabbit but they were not reproducible in the conventional rabbit or other animals. The method is instructive for investigations on qualitative characteristics of glycogen in animals but not for systematic studies in normal or experimental animals. The mode of action of DMSO remains enigmatic.

Proposed Course of the Project: This project has been completed and the manuscript is being prepared for publication.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02284-05 LNNS
PERIOD COVERED October 1, 1980, to September 30, 1981		
TITLE OF PROJECT (80 characters or less) Improvement of current methods of fixation by perfusion for preservation of glycogen		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: J. Cammermeyer                      Guest Worker                      LNNS NINCDS		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Neuropathology and Neuroanatomical Sciences		
SECTION Office of the Chief, LNNS		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0	PROFESSIONAL: 0	OTHER: 0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) This project has been completed and the resulting manuscript has been published.  Cammermeyer, J., and Fenton, I.: Improved preservation of neuronal glycogen by fixation with iodoacetic acid-containing perfusates. <u>Exp. Neurol.</u> 72: 429-445, 1981.		







## ANNUAL REPORT

October 1, 1980 through September 30, 1981

Laboratory of Neural Control, Intramural Research Program  
National Institute of Neurological and Communicative Disorders and Stroke

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## ANNUAL REPORT

October 1, 1980 through September 30, 1981

Laboratory of Neural Control, Intramural Research Program  
National Institute of Neurological and Communicative Disorders and Stroke

Robert E. Burke, M.D., Chief

### Introduction

Research work in the Laboratory of Neural Control (LNLC) is devoted largely to studies of the central and peripheral neural mechanisms involved in the control of movement in mammals. Emphasis is on neural organizations at the final stages of motor output - i.e., those contained within the spinal cord and regions of the brain stem and cerebral cortex that project directly to the spinal cord. A variety of technical approaches are used, including conventional electrophysiological, neuroanatomical and histochemical methods, as well as novel techniques (many developed within LNLC) for recording neural and kinesiological data from awake, intact animals (cats and monkeys) during the production of normal motor behaviors. Many of the latter are derived from the long-standing interest of LNLC staff members in the development of neural prostheses to aid neurologically handicapped patients.

### Present Organization

During FY 1981, the staff of the Laboratory of Neural Control (LNLC) has consisted of up to 14 investigators, including four permanent senior scientists, seven post-doctoral fellows, and three Guest Workers. The permanent staff also includes three senior support personnel (two engineers and one physiologist), the laboratory secretary, and an animal caretaker/biological technician. The non-permanent, part-time staff includes one Laboratory Aide and a student computer programmer. The staff members of LNLC have, in various combinations, backgrounds in neurophysiology, clinical medicine and neurology, and in biomedical engineering and computer sciences. Several staff members also have considerable expertise with biomaterials and techniques for fabrication of devices designed for chronic implantation. Because of the close interaction and collaboration within the Laboratory, LNLC is not divided into formal Sections but the research effort can be described under four general headings, divided roughly by methodological approach:

1. Research involving more or less conventional electrophysiological techniques and directed toward clarifying aspects of the cellular physiology and neuronal circuitry operating in the control of movement at the spinal cord level. This work is done largely using acute reduced preparations (both cats and monkeys), either anesthetized or unanesthetized after brain destruction under volatile gas anesthesia. Some phases of this work also involve neuro-anatomical techniques of cell labeling and pathway tracing with the exogenous protein tracer horseradish peroxidase (HRP), while other aspects involve study of muscles with conventional methods for muscle fiber histochemistry.
2. Research projects that utilize novel methods for recording the activity of individual neural elements in the central and peripheral nervous systems of awake, intact animals (both cat and monkey) that are either free to move or able to perform specific motor tasks with minimal restraint. This work utilizes techniques for recording kinesiological data (limb position, joint angles, etc.) from videotape records and/or from chronically-implanted transducers.

3. Theoretical and computer modeling studies of information processing in neural networks and development of general approaches that optimize analysis of multichannel data streams that can be recorded from ensembles of neural elements. The latter is extremely important in systematic studies of neural and mechanical events during motor behaviors of freely-moving animals, in which movements and associated data streams often exhibit considerable variability.

4. Activities concerned directly with the development of new instruments and techniques, and the further refinement of existing methods, for recording and analyzing neurally-relevant data from intact, freely moving animals.

#### Project Summaries:

The functional output elements of the motor system are the motor units (a spinal motoneuron plus the set of muscle fibers, or "muscle unit", innervated by it). The central nervous system controls movement by grading the numbers and identities of active motor units (the process of "recruitment") and by regulating the frequency of their firing (referred to as "rate coding"). The project entitled "Intrinsic Properties of Motor Units" is designed to produce comprehensive descriptions of the electrophysiological, mechanical, morphological and histochemical characteristics of motor units in unit populations that make up heterogeneous muscles in the cat hindlimb. Previous work in LNLC has suggested that the unit types present in the normal, fully mature MG motor pool are relatively stable and resistant to alteration of unit type characteristics when challenged by conditions designed to modulate overall usage patterns within the physiological range for the muscle in question. Experimental work on this project during FY 1981 has been limited to completion of data analysis of experiments done previously on the effect of chronic (duration about 6 months) thoracic spinal cord lesions (hemisections or complete spinal transections) on the properties of motor units in the medial gastrocnemius (MG) muscle of adult cats. Spinal hemisection produced mild persistent hyperreflexia, with minimal functional impairment and correspondingly little alteration in the properties of the whole MG muscle or its motor units. However, complete spinal transection produced (as expected) chronic paraplegia with marked hyperreflexia, spasticity, and muscle atrophy. In contrast to earlier models of muscle atrophy, spinal transection produced some alterations in the proportions of the different motor unit types, and in the histochemical fiber types associated with them. These changes, and details of the alterations in properties of units in each of the major motor unit types, were rather different from findings in the equally marked atrophy that occurs after chronic limb immobilization. Explanations are not obvious but the conditions are clearly different in terms of their effects on spinal reflex mechanisms. A probabilistic sampling model, developed to facilitate interpretation of these data, supports the intuitive expectation that, for experimental samples of practical size, deviations from a normal motor unit composition must be quite large in order to be detectable.

The project entitled "Motor Control Systems in the Spinal Cord" is closely related to the above, in that the organization of neural mechanisms present in, and projecting to, spinal cord segments is often correlated with motor unit type. During FY 1981, four aspects were studied. We completed a project designed to analyze the supraspinal control of a polysynaptic segmental pathway that conveys excitatory synaptic information from low threshold distal skin afferents (mainly in the sural nerve) to ankle extensor motoneurons, primarily those innervating fast twitch muscle units (both types FF and FR). This pathway



can alter the balance of functional thresholds to favor recruitment of the fast twitch units that are normally relatively high threshold. Spinal cord interneurons in this excitatory cutaneous pathway receive convergent excitation from both rubrospinal and corticospinal tracts, suggesting that these sources of supraspinal motor commands may also exert differential control of the functional thresholds of slow twitch, normally low threshold, and fast twitch, normally higher threshold, motor units.

A second aspect of this project concerns a study of the detailed anatomy of muscle stretch receptor (group Ia) afferents and of the contacts they establish on defined types of  $\alpha$  motoneurons, using intracellular iontophoretic injection of horseradish peroxidase (HRP) into functionally identified group Ia afferents and subsequently into type-identified motoneurons. This sub-project has continued over several years and is now reaching completion. Some 20 functionally-identified combinations of afferent and  $\alpha$ -motoneuron have now been fully reconstructed at the light microscope level, providing illustrations of a very wide range of anatomical configurations, from relatively simple systems that involve a few boutons located within a confined region of the motoneuron dendritic tree, to extremely complex, widely dispersed systems. Such results fit well with earlier electrophysiological observations from this and other laboratories that suggested that Ia afferent contacts are established mainly on motoneuron dendrites, including their most distal regions. Because the  $\alpha$ -motoneurons are identified according to unit type, this material is also being analyzed to provide detailed data on the anatomical specializations that correlate with motor unit type.

Related to the above is a project begun during FY 1981 on post-tetanic potentiation (PTP) of monosynaptic group Ia excitatory synaptic potentials (EPSPs) in MG  $\alpha$ -motoneurons. We have confirmed findings of another group that the percent increase in EPSP amplitude following a standard conditioning tetanus is negatively correlated with initial (control) EPSP amplitude and, much less strongly, with motoneuron input resistance. Additional studies suggest that the negative correlation can be explained on the basis of non-linear effects of voltage perturbations at the dendritic sites at which most group Ia synapses terminate. Our data are consistent with a model for PTP that involves a complex sequence of depression and facilitation of transmitter liberation, such as occurs at the much more accessible neuromuscular junction. This study is continuing with an analysis of the interaction of PTP with conditioning stimuli that produce primary afferent depolarization and presynaptic inhibition.

A final aspect of this project that began in late FY 1981 is designed to investigate the organization of synaptic input systems that project to motoneurons of the flexor digitorum longus (FDL) and flexor hallucis longus (FHL) muscles. These muscles have been regarded as "synergists" because they have identical mechanical actions, they lie parallel to one another in the leg and their tendons join into one, their motoneurons share the same motor cell column in the spinal cord. However as reported in the Annual Report for FY 1980, FDL and FHL exhibit dramatically different activity patterns in freely moving cats, especially during locomotion, when FDL is active primarily in the swing phase and acts as a functional flexor whereas FHL acts as a typical antigravity extensor. Preliminary results using intracellular recording and electrical stimulation of a wide variety of peripheral nerves suggest that some polysynaptic systems make quite different connections with the FDL and FHL motoneurons but much more work is necessary before the functional disparity between these anatomical synergists can be explained. It seems useful to point out that this project, which requires analysis by more or less conventional

electrophysiology, stems directly from observations made in freely-moving animals with novel techniques, which are discussed below.

The information and conceptual models obtained from neurologically reduced preparations must be tested and supplemented by examination of neural activity in the intact, freely moving animal that exhibit purposive motor behaviors. Approaches to this important question are represented in the project entitled "Neuron Activity in Locomotion". In the past, much of this project was devoted to development of techniques that would permit recording of activity in individual neural elements in freely moving cats, using chronically implanted electrodes in conjunction with other devices (electromyographic electrodes and length and force transducers) designed to monitor activity in individual muscles. In addition, it is necessary to record the details of movements emitted by experimental animals, using videotape equipment. Meaningful analysis of such temporally correlated multiple channel data streams requires not only sophisticated equipment but also new conceptual frameworks. The systems available in LNLG at present are now sufficiently developed to permit relatively reliable recording from functionally-identified sensory afferent neurons, as well as from identified alpha motoneuron axons in ventral roots, using chronically-implanted semi-microelectrodes.

For technical reasons, this work centers on studies of the muscles that extend the knee and for the past several years the activity patterns in identified muscle receptor afferents (muscle spindle and tendon organ afferents) have been of primary interest. A central question is how the  $\gamma$  motor system, which modulates muscle spindle sensitivity, participates during a variety of motor behaviors. This can be inferred from the activity in functionally-identified primary and secondary muscle spindle afferents, provided accurate information is available about length and electrical activity in the target muscles. The technical complexity of these experiments and the fact that clearly identifiable afferents are encountered at random make progress relatively slow. However, the results to date continue to suggest that the  $\gamma$  motor system operates in much more complex ways during normal movement than had been envisioned from conventional reflex physiology. The activity of spindle afferents in some flexor muscles that shorten rapidly during locomotion suggest that their  $\gamma$  motoneurons are co-activated with the  $\alpha$  motoneurons, while other spindle afferents, particularly in those extensor muscles that undergo lengthening activation in stepping, exhibit less evidence of fusimotor bias. During FY 1981, a method was developed for perfusing muscle nerves with dilute local anesthetics via implanted nerve cuffs, which permits selective paralysis of fusimotor axons. The effects of such artificial modulation of fusimotor effects have confirmed the presence of substantial fusimotor bias in some muscles during particular phases of the step cycle. The observed complexities of dynamic spindle afferent action during normal movements will permit refinement of quantitative models of fusimotor activity patterns and we have begun to implement such modelling on the PDP10 available in DCRT.

A closely related project is recording discharge patterns of individual motor axons from the ventral roots of freely-moving cats. In many cases, both afferents and efferent axons are recorded in the same animals. This represents the first technically feasible method for obtaining such motor unit activity patterns from intact, behaving animals. Axonal conduction velocities can be measured using special nerve cuff electrodes and the muscle of destination is determined by spike-triggered averaging of electromyographic activity. In favorable cases, the same axon being recorded can also be stimulated by brief, small amplitude pulses delivered through the recording electrode (identified as

the same axon by conduction velocity and EMG waveform criteria), making it possible to identify motor unit types using the criteria developed for cat motor units in anesthetized animals. This represents the only currently feasible direct method for study of recruitment patterns in relation to motor unit type in otherwise fully-characterized animal muscles. One striking and unprecedented result thusfar is that motor units within the same anatomical muscle (specifically the sartorius, a combined hip flexor and knee extensor) can be shown to be specialized for either flexor or extensor function even though the muscle units can be recorded at a single location within the muscle. This, together with the observation of disparate function between the anatomically proximate FDL and FHL motor nuclei, suggest highly precise mechanisms that direct the organization of synaptic inputs to particular species of motoneurons.

Two new subprojects were begun in FY 1981. The first represents an extension of studies noted above of the dynamic activity in ankle and toe muscles during locomotion and other motor behaviors, especially those for which motor unit population data and detailed histochemical analyses are either available or under study within LNLN. This has confirmed earlier observations about the distinctive activity patterns in the FDL and FHL muscles, and will provide similar detailed observations on other muscles whose functions are at present poorly documented. A second project initiated in FY 1981 is a study of plasticity in ascending somatosensory systems. This is intended to follow up on recent evidence for widespread reorganization of somatotopic responses in areas 1 and 3b of the primary somatosensory cortex in monkeys after lesions of selected peripheral nerves or dorsal roots. The site of such reorganization is unknown but it seems possible that it may occur in the spinal cord or at the second order sensory neurons in the dorsal column nuclei. We have begun preliminary studies of the feasibility of using chronically-implanted microwire electrodes in the dorsal column nuclei to record the sensory fields of small groups of second order cuneate neurons before and after nerve or dorsal root lesions. Cats with implanted nerve cuff electrodes are used to permit precise identification of units recorded. If it indeed proves possible to utilize the chronic recording techniques developed in LNLN for this approach, it will represent a major advance in research on "plasticity" of function within the mammalian CNS.

The project entitled "Cortical Mechanisms of Voluntary Motor Control" is concerned mainly with studies of neural activity in particular regions of the cerebral cortex with proximate output to the spinal cord (the sensorimotor cortex and supplementary motor area) during voluntary motor behavior in awake intact animals. Both cats and monkeys are being studied. During FY 1981, studies of the forearm and hand region of the monkey motor cortex continued, using movable microelectrodes, intracortical microstimulation (ICMS) and signal averaging methods, to study the interrelations between highly localized cortical regions and particular forearm muscles. Monkeys are trained to produce stereotyped forearm movements in a visual tracking task, and the properties of task-related cortical neurons, identified as to whether or not they project through the pyramidal tract, are evaluated. Patterns of sensory responsiveness of task-related and unrelated cells are also studied. The cortical "colonies" of neurons that are correlated with activity in particular muscles or muscle groups can be very extensive and often are discontinuous. ICMS in such regions can produce complex mixtures of excitation and inhibition at the spinal output level. As part of this study, the histochemical composition of the major wrist extensors and flexor muscles have been analyzed in several species of laboratory primates. Of particular interest is the flexor carpi ulnaris, which has a



complex internal structure and very different histochemical mixtures in its two major heads. Detailed studies are planned of the cortical control of these heads during task-related movements, using chronically implanted EMG and force recording devices.

Evaluation of an improved capacitor stimulating electrode, suitable for chronic implantation in the cortex of deep structures, was undertaken in FY 1981, in collaboration with members of the Neural Prosthesis Program of NINCDS. Miniaturized capacitor electrodes can be used for chronic intracortical stimulation in monkeys with much less danger of tissue damage than with conventional noble metal electrodes. These results are relevant to current attempts to develop safe and practical sensory prostheses that involve direct stimulation of CNS tissue.

A subproject using chronically-implanted microelectrodes in the sensorimotor cortex of cats during locomotion and postural activity was completed in FY 1981. The results show that pyramidal tracts neurons sharing the same sensory input can behave quite differently during locomotion, depending on cortical location (and presumably, on the destination of their axons). There is evidence suggesting the existence of gating of sensory inflow to the motor cortex during the late swing ( $E_1$ ) phase of stepping, and during certain phases of landing from short vertical drops. Such results are very difficult to interpret because of the complexity of responses and the difficulty in controlling all of the associated variables in intact animals. However, they illustrate the importance of exploring such questions in intact, behaving animals, since the required information can be gathered in no other way.

Work under the project entitled "Models of Neural Interaction" has concerned the fundamental problem of pattern detection by ensembles of neurons and the more applied problem of extracting information about neural activity from multichannel data streams such as are obtained from intact, moving animals. The work has taken several avenues. Considerable effort is being expended to develop a general strategy for handling multichannel data streams during repetitive movements of different durations, such as occur during locomotion, as well as the much more difficult problem of dealing with non-stereotyped but behaviorally significant movements that are not strictly repeatable. As a result, a library of computer-based data analysis systems are being developed that will permit LNLC staff members to utilize the Laboratory's new computer systems to reduce the voluminous data streams that emerge from chronic-implant experiments. Data output on muscle receptor activity during locomotion, described above, is being used to develop a computer model of fusimotor-skeletomotor interaction during locomotion by comparing the properties of stretch receptors, gathered from functionally reduced preparations, with the activity of the same types of receptors in intact, moving animals. In addition, the theoretical basis for extraction of neural activity information from multichannel neuroelectric recordings has been refined (Independent Source Theory) and, at the appropriate time, will be tested using firing patterns of alpha motoneurons and EMG recordings as the test system.

One member of the Laboratory staff has continued a collaboration with the Department of Otolaryngology, University of California, San Francisco, on a new model of pitch perception that is directly relevant to the development of a feasible auditory prosthesis for hearing-impaired patients. This model suggests that pitch information may be extracted by neurons in the medial superior olive that respond to very small differences in input timing arising because of mechanical traveling waves that excite relatively restricted regions of the

cochlear basilar membrane. Testing of the model in acute animal experiments is being concluded and the project should be concluded by the end of FY 1981.

Work done under the project entitled "Techniques for Making Contact with the Nervous System" largely results from the needs and demands generated by other projects in LNLC, although some input is received from outside groups in terms of questions or specific fabrication needs. During FY 1981, refinement of several chronic implant systems has continued, including incorporation of a system to deliver controlled amounts of local anesthetics within nerve cuff electrodes to permit modulation of fusimotor bias during movement in intact animals. The "map-pin" electrode design, developed some years ago for recording from the motor cortex of monkeys, has been refined to permit its use in the cuneate nucleus of cats, where movement problems are much more severe. A new treadmill system for cats is nearing completion. This has been a very complex technical problem because of the need for mechanical flexibility, high belt speed, and minimum electrical and auditory noise. A new system for producing controlled postural movements in cats is under development to permit study of neural and muscle activity during postural readjustments. A major problem with multiple channel recording from moving animals is the cable system used to lead the signals from the animal. Telemetry is impractical for more than 2 or 3 data channels and some of the locomotion projects require up to 32 channels. We have developed a printed circuit cable with integrated preamplifiers for this application which has been highly successful in minimizing electrical noise in high gain signals. We have continued to refine a modular series of electronic signal-processing instruments which are applicable to many problems in LNLC. Because of their flexibility and modular design, these instruments can be used in all of the experimental setups within the Laboratory, leading to considerable savings in equipment outlay and maintenance. Many of the methods developed in LNLC in the course of its own projects have been of general interest and staff members identified with this project serve as sources of information for inquiries from around the world. An informal newsletter about matters relevant to biomaterials and chronic implantation of various devices has been circulated for several years and now reaches about 100 individuals worldwide, at their request. Evaluation of the state of the art in biomaterials, electrode wires, insulations and electronic and electromechanical devices applicable to motor systems research and/or motor prosthesis applications continues constantly in LNLC and this information is made available to other groups at NIH and elsewhere.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  ZOI NS 01686-13 LNLC
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Motor Control Systems in the Spinal Cord		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Robert E. Burke, M.D. Other: James W. Fleshman, Ph.D. Loyd L. Glenn, Ph.D. Aharony Lev Tov, Ph.D. Martin J. Pinter, Ph.D.	Chief Guest Worker Guest Worker Visiting Fellow Guest Worker	LNLC LNLC LNLC LNLC LNLC
		NINCDS NINCDS NINCDS NINCDS NINCDS
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Neural Control		
SECTION		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.1	PROFESSIONAL: 0.8	OTHER: 0.3
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) This project is designed to provide information on the mechanisms operating within <u>reflex</u> systems in the spinal cord, which include <u>alpha motoneurons</u> as the output link, as well as on the interconnections and interactions between reflex pathways and control systems descending to the spinal cord from supraspinal centers. Particular consideration is also given to correlations between <u>synaptic organization</u> , intrinsic neuronal properties, and dynamic behavior of the <u>alpha motoneurons</u> and the motor unit type, defined by the <u>physiological</u> characteristics of the innervated <u>muscle fibers</u> .		

## Project Description:

Objectives: This project is designed to provide information about the organization of neuronal systems in the mammalian spinal cord which ultimately control the activity patterns of motor units (motoneurons and the muscle fibers they innervate). Topics of interest include analysis of reflex pathways within the spinal segment and the interaction between primary afferent and supraspinal descending systems in the control of information flow in spinal segmental motor mechanisms. Of particular interest is the organization of synaptic input systems that project to motor units of different types within particular motor pools.

Methods Employed: A variety of experimental approaches have been used in this project but all are applied to study the lumbosacral spinal cord of the adult cat. Much of the work has been done on animals anesthetized with barbiturate,  $\alpha$ -chloralose or inhalation (Halothane) anesthesia, or on unanesthetized animals following destruction of the supratentorial brain under rapidly-reversible inhalation anesthesia (decerebrate preparations). Most of such experiments have been devoted to intracellular recording from identified alpha motoneurons using conventional micropipette electrodes, with analysis of synaptic potentials produced by electrical stimulation of peripheral nerves and/or of selected supraspinal structures (e.g., red nucleus, reticular formation, motor cortex, etc.) using stereotaxically placed electrodes. In addition, we are using the method of intracellular iontophoresis of the tracer protein, horseradish peroxidase (HRP), to permit neuroanatomical study of functionally-identified neuronal elements in the spinal cord. In such cases, animals are perfused transcardially with fixative under deep anesthesia, and spinal cords are then dissected, photographed and processed for demonstration of HRP (diaminobenzidine - cobalt method) in frozen- or Vibratome-sectioned material.

## Major Findings:

### A. Supraspinal Control of Cutaneous Excitation in Extensor Motoneurons.

Work on this subproject, demonstrating rubrospinal and corticospinal tract control of transmission of information in the interneuronal pathway carrying excitatory input from low threshold cutaneous afferents from distal hindlimb to ankle extensor motoneurons, was completed in FY 1981. This cutaneous pathway is of particular interest because it tends to excite motoneurons of fast twitch (types FF and FR) motor units much more than the cells of slow twitch (type S) units. Intracellular recordings were obtained from ankle extensor motoneurons, mainly medial gastrocnemius (MG) as identified by the pattern of group Ia excitation projecting to them. Synaptic potentials produced by electrical stimulation of the ipsilateral sural (SUR) nerve were recorded with and without conditioning stimulation (short trains of pulses) to the contralateral red nucleus or to the medullary pyramid via stereotaxically-placed monopolar electrodes. The critical records were obtained by computer averaging and a variety of controls were used to ensure that supraspinal stimuli were confined to the appropriate structures.

Synaptic potentials produced by the SUR nerve in MG motoneurons are composites of excitatory and inhibitory components. The earliest response is an excitatory component (EPSP) with central latency of 1.8 to 2.5 msec (mean latency about 2.0 msec), while the first order interneurons in the dorsal horn begin to respond with a minimum latency of 0.5 msec. This indicates the presence of at least two interneurons interposed between the primary afferents and the motoneurons. The inhibitory (IPSP) components have a minimum central latency about 1 msec longer than the EPSP component, suggesting the presence of a third interneuron in the minimum inhibitory pathway. SUR EPSPs are produced by the lowest threshold afferents in the SUR nerve (<1.2 times electrical threshold) while the apparent threshold for IPSP components is slightly higher. EPSP components appear fully developed with stimuli <3xT but IPSPs continue to increase with higher stimulus intensities. Conditioning stimulation of either rubrospinal or pyramidal tract axons facilitates transmission of EPSP and IPSP components, suggesting that these descending systems excite, directly or indirectly, at least some of the same segmental interneurons that convey the SUR effects to the motoneurons. The functional "meaning" of this convergence remains to be determined but the existence of descending control of the cutaneous excitatory pathway in principle provides an important degree of flexibility to the control of motor unit pools. The organization of the convergent descending/cutaneous excitatory system could provide an excitatory drive to motor units at the high threshold end of the recruitment spectrum so as to promote synchronous activation of an entire motor unit pool, as occurs in rapid, forceful movements such as jumping or gallop.

#### B. Anatomy of Muscle Afferent - Motoneuron Relations.

Experiments involving injection of the tracer protein horseradish peroxidase (HRP) into functionally identified group Ia afferents and into  $\alpha$ -motoneurons postsynaptic to them (identified as to motor unit type) began in FY 1978 and have been noted in previous Annual Reports. During FY 1981, we have continued to accumulate reconstructions of the complete trajectories of group Ia afferent collaterals, and of the contacts they appear to make on the dendrites of physiologically-identified motoneurons. Tissue processing and reconstruction methods have been standardized using transparent photomicrographic montages of each section on high contrast negative material at relatively high magnification, permitting overlay matching of structures in serial sections. This obviates the need for tracing highly complicated, intertwined structures under the camera lucida and permits reliable mapping and identification of every labeled element in each section. The "contacts" established by labeled afferents on labeled postsynaptic structures are defined in this work by examination under oil-immersion magnification but these may or may not be functional synapses. Corroborative evidence can only be obtained by subjecting each contact to ultrastructural study but this is unfortunately impractical at present. Our current view is that the contacts seen at the light microscope level represent maximum estimates for the number of functional synapses on any given motoneurons and that the actual number of these must be less than or equal to the observed number.

Our experience to date indicates that there may sometimes be considerable overlap between the territories of neighboring group Ia collateral

arborizations, and that several collaterals can make synaptic contact with a given motoneuron, often on spatially separate regions. Most of the observed contacts occur in the motoneuron dendritic tree with multiple contacts on different dendrites at quite variable physical and, presumably, electrotonic distances from the motoneuron soma. A striking finding is that a given Ia collateral usually gives rise to two to three major collateral branches in the dorsal horn, each of which can contribute contacts to a given motoneuron. Thus, the first branch points of consequence to a given motoneuron are those that form in the dorsal horn, one to two millimeters from the motoneuron. The available material is still quite limited because of the painstaking work required for each reconstruction. However, in the 14 homonymous Ia contact systems thusfar reconstructed, the number of Ia boutons ending on individual motoneurons has varied from 4 to 34, with a median number of 10, while the 5 heteronymous systems have varied from 3 to 10 contacts, with a median of 5. The material is too limited to answer whether or not there is a systematic difference in the number or the average density of Ia contacts in relation to postsynaptic motor unit type. To achieve statistical significance, we estimate that more than 100 total reconstructions would be necessary to answer this question, which is impractical given the large effort needed for each one. This aspect of the project will conclude in FY 1982 with accumulation of some material suitable for ultrastructural analysis through collaborative arrangements.

#### C. Post-tetanic Potentiation of Group Ia EPSPs.

During FY 1981 we began a subproject designed to evaluate the characteristics and mechanism underlying post-tetanic potentiation of group Ia monosynaptic EPSPs in ankle extensor motoneurons, primarily in the medial gastrocnemius (MG) motor nucleus. The amplitudes of composite homonymous Ia EPSPs produced by electrical stimulation of the MG muscle nerve have been studied before ( $V_C$ ) and after ( $V_P$ ) conditioning tetani of various frequencies and durations in pentobarbital-anesthetized cats. Another laboratory recently reported that the degree of PTP in Ia EPSPs varies inversely with control EPSP amplitude and with the input resistance ( $R_N$ ) of the postsynaptic motoneurons. We have confirmed these results, in that the change ( $V_P/V_C$ ) in EPSP amplitude produced by a standard tetanus (500 Hz for 20 seconds) varies inversely with  $V_C$  ( $r = -0.5$ ;  $p < 0.001$  by 2-tailed  $t$  test) and less strongly but nevertheless inversely with  $R_N$  ( $r = -0.33$ ;  $0.02 < p < 0.01$ ), but both correlations in our material are much less strong than in the original report. The interpretation of PTP data is complicated by the existence of post-tetanic depression of EPSPs in addition to potentiation. The presence of depression is indicated by the fact that Ia EPSPs recorded immediately after a prolonged tetanus are often smaller than control EPSPs and the amplitude then reaches a peak greater than  $V_C$  some 15 - 25 seconds after the end of the tetanus. There is a strong positive correlation between the amplitude of the first EPSP post-tetanus ( $V_1/V_C$ ) and the amplitude at PTP peak ( $V_P/V_C$ ). Thus, any observed "maximum" PTP represents the balance between depression and potentiation phenomena that have been analyzed in detail at neuromuscular synapses, but much less completely in synapses within the mammalian CNS. The observed post-tetanic depression could represent several phenomena, including possible failure of action potential invasion into the complex Ia arborizations seen in our anatomical work,



depletion of transmitter released from individual boutons, and perhaps alterations in the factors that cause transmitter release (e.g., kinetics of  $\text{Ca}^{2+}$  entry and sequestration in synaptic terminals). We have no evidence consistent with post-tetanic failure of action potential invasion, since the amplitudes of Ia volleys entering the cord and of Ia arborization potentials recorded in the ventral horn were maximal immediately post-tetanus. Double pulse test stimuli (250 msec interval) were used to assess depression due to transmitter and release factor kinetics which appear to explain the fact that the amplitude of the second pulse in such pairs is negatively correlated with the amplitude of the first when studied at the neuromuscular junction. After PTP, the disparity between second and first EPSPs in test pairs increases markedly, suggesting that PTP must involve at least some element of increased transmitter release from individual group Ia synaptic boutons, as occurs at the neuromuscular junction. The degree of this synaptic depression was not correlated with  $V_C$  or  $R_N$ , suggesting that explanations for the weak correlations between these variables and  $V_p/V_C$  must lie elsewhere. Taking into account the above experimental observations and theoretical considerations of PSP summation in dendritic neurons, the available evidence suggests that the weak correlations between the degree of PTP and both EPSP amplitude and motoneuron input resistance are due simply to factors of non-linear summation of voltage perturbations in postsynaptic structures with different local input resistances. This non-linearity results from the fact that PTP appears to involve increased conductance changes at individual synaptic sites, many of which are in dendrites where local input resistances are high and consequent voltage perturbations are large. Under these conditions, doubling conductance produces much less than double the voltage change, because the driving potential for the synaptic process is fixed. The degree of this non-linearity should increase as composite EPSP voltage and/or motoneuron input resistance increase. Systematic modeling studies are underway to assess this explanation more quantitatively.

#### D. Synaptic Organization in FDL and FHL Motor Nuclei.

In the Annual Report for FY 1980, we reported the observation that the dynamic activity patterns of the flexor digitorum longus (FDL) and flexor hallucis longus (FHL) muscles are quite different when studied in intact cats during normal locomotion (see also FY 1981 report of Project Z01 NS 02080-08 LNLc). The FHL muscle has a stereotyped antigravity extensor function while the FDL is active primarily in a short burst during the early flexion (swing) phase of the step cycle. FDL also exhibits remarkable bursts of "facultative" activity during perturbed step cycles which appear to be corrective and these are not echoed in FHL or in any of the other limb muscles studied simultaneously. These observations are of considerable interest because FDL and FHL are strict anatomical synergists with common tendons of insertion and identical mechanical action (plantar-flexion of the distal phalanges), and their motoneurons share the same motor cell column in the spinal cord. FDL motoneurons receive monosynaptic group Ia EPSPs from FHL Ia afferents as well as from FDL, and the converse is true for FHL cells. Because of the facts, these two muscles have always been regarded as strict "synergists", yet they are clearly not synergists in dynamic action. We have begun to study possible



differences in the organization of synaptic input to FDL and FHL  $\alpha$ -motoneurons in order to define those systems that might produce the two disparate functional patterns. Initial experiments in Nembutal-anesthetized or in unanesthetized, anemically decerebrate cats have disclosed that FHL motoneurons receive stronger group Ia projections, on the average, than do FDL cells, both from homonymous and heteronymous sources. Furthermore,  $\alpha$ -motoneurons of another major toe plantar-flexor, the plantaris, receive stronger group Ia EPSPs from FDL than FHL. Polysynaptic PSPs produced by ipsilateral hindlimb nerves from distal skin regions also tended to include predominant excitatory components in FDL motoneurons, while such excitation was much weaker or absent in most FHL cells. Electrical stimulation of the ipsilateral forelimb plantar pad, or the superficial radial nerve, produced polysynaptic IPSPs in most FHL motoneurons which were entirely absent in almost all FDL cells. These preliminary results suggest that the organization of synaptic input to FDL and FHL motoneurons is indeed different in several respects but the information is still too limited to suggest "reasons" for the observed functional differences. However, the prominence of polysynaptic excitation from distal limb skin sources in FDL motoneurons may be connected with their apparent sensitivity to perturbations in step cycles.

#### Significance to Biomedical Research and the Program of the Institute:

Active movement of mammals in space is accomplished by motor units with motoneurons located in the spinal cord. Analysis of the central nervous system control of movement requires a detailed understanding of the organization and interaction of input systems to the spinal cord segments, both from peripheral afferent sources and from supraspinal structures. There is now considerable evidence for the existence of functional specializations among the muscle fibers of different motor unit types, as well as among their  $\alpha$ -motoneurons. The long-range goal of the present project is to analyze the patterns of neuronal organization present in the spinal cord as they relate to the motor unit types in order to further our understanding of how motor units, and therefore movements, are controlled. The specializations evident in different motor unit types has long been thought to be associated with equivalent differences in functional usage. Definition of synaptic organization and direct study of motor unit usage patterns in normal movements are necessary to test this hypothesis, as are direct studies of dynamic muscle usage in intact animals. The latter type of investigation has brought up additional questions about the organization of synaptic input to different motor pools, as exemplified by the current work on the functional differences between the FDL and FHL pools. Such studies are of clear relevance to analyses of both normal and abnormal movement patterns in man. They also bear importantly on the interpretation of results of clinical investigations using electromyography and muscle histochemistry in normal human subjects and in patients with neurological diseases.

#### Proposed Course of the Project:

The work on the interaction of primary afferent and descending control of transmission in the excitatory cutaneous pathway from distal skin to ankle extensor motoneurons was completed in FY 1981. During FY 1982, we intend to

complete the data base on the detailed anatomy of group Ia afferent collaterals, and on the anatomical structure of alpha motoneurons of identified motor unit type. An attempt will be made to process some of this material in such a way as to be suitable for both light and electron microscopic study, even though no firm arrangements have been made for collaborative ultrastructural study, which should ideally involve study of serial sections. The study of post-tetanic effects on group Ia EPSPs will continue with special emphasis on the interaction of post-tetanic effects with the effects of conditioning stimuli that are known to produce primary afferent depolarization and presynaptic inhibition. Such observations should throw new light on the mechanisms of both PTP and presynaptic inhibition. The study of synaptic organization in the FDL and FHL motor nuclei will continue with attempts to study motoneuron firing patterns and underlying synaptic events in decerebrate animals during induced "fictive" locomotion.

#### Publications:

Burke, R. E. Intracellular recording and stimulation of mammalian CNS neurons with micropipette electrodes. In: Electrophysiological Techniques. Bethesda, MD: Society for Neuroscience, 1979. pp. 77-91.

Burke, R. E. Motor unit recruitment: What are the critical factors. In Desmedt, J. E. (Ed.): Recruitment Patterns of Motor Units and the Gradation of Muscle Force. Progress in Clinical Neurophysiology, Vol. 9 Basel: Karger 1981, pp 61-84.

Burke, R. E. Motor units: Anatomy, physiology and functional organization. In Brooks, V. B. (Ed.): Handbook of Physiology. Section 1. The Nervous System. Vol. II, Part 1 Motor Control. Washington, DC: American Physiological Society. 1981. pp. 345-422.

Glenn, L. L. and Burke, R. E. A simple and inexpensive method for 3-dimensional visualization of neurons reconstructed from serial sections. J. Neurosci. Methods. In press, 1981.

Pinter, M. J., Burke, R. E., O'Donovan, M. J. and Dum, R. P. Supraspinal facilitation of cutaneous polysynaptic EPSPs in cat medial gastrocnemius motoneurons. Exp. Brain Res. In press, 1981.

## PERIOD COVERED

October 1, 1980 to September 30, 1981

## TITLE OF PROJECT (80 characters or less)

Techniques for Making Connections with the Nervous and Musculoskeletal Systems

## NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	Martin J. Bak	Electronics Engineer	LNLC	NINCDS
Other:	Lawrence D. Abraham, Ph.D.	Guest Worker	LNLC	NINCDS
	George M. Dold	Engineering Technician	LNLC	NINCDS
	Joaquin A. Hoffer, Ph.D.	Senior Staff Fellow	LNLC	NINCDS
	Gerald E. Loeb, M.D.	Medical Officer (Res.)	LNLC	NINCDS
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	Claude I. Palmer, Ph.D.	Visiting Fellow	LNLC	NINCDS
	Martin J. Pinter, Ph.D.	Guest Worker	LNLC	NINCDS
	Edward M. Schmidt, Ph.D.	Research Physiologist	LNLC	NINCDS
	Frederick T. Hambrecht, M.D.	Head, Neuroprosthesis Program	FNP	NINCDS

## COOPERATING UNITS (if any)

Fundamental Neurosciences Program, NINCDS

## LAB/BRANCH

Laboratory of Neural Control

## SECTION

## INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Maryland 20205

## TOTAL MANYEARS:

2.0

## PROFESSIONAL:

0.6

## OTHER:

1.4

## CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS☐ (b) HUMAN TISSUES☒ (c) NEITHER☐ (a1) MINORS ☐ (a2) INTERVIEWS

## SUMMARY OF WORK (200 words or less - underline keywords)

This project is intended to develop techniques for the acquisition and processing of neuroelectric signals from the central and peripheral nervous system in acute and chronic neurophysiological preparations. Because of this laboratory's continuing interest in sensorimotor neural activity during unrestrained movements, the project also includes development of chronically implantable mechanical transducers, catheters, and connectors.

## Project Description:

Objectives: Successful monitoring of neural activity from the peripheral or central nervous system in intact, moving animals requires the development of novel techniques that apply to unique recording situations. The latter include recording from both acute and chronic preparations in which stable and discriminable single unit activity is required, but the problems are particularly critical in recording from animals that are awake, comfortable and moving freely or with minimal restraint. Delivery of functionally effective stimuli through metal microelectrodes without damaging the electrode or causing pathological changes in nearby neurons has now become essential for ongoing laboratory experiments, and this requires considerations different from those involved in recording. This project is designed to evaluate methods, materials and instrumentation designs to solve particular research problems in both recording and stimulation situations.

Methods Employed:

## A. Evaluation of materials suited for biological implantation.

The evaluation of the physical properties and biocompatibility of Parylene-C, metallic iridium, tantalum metal as well as Ta-TaO<sub>5</sub> insulation, and certain medical grade silastic rubbers has continued, primarily through examination of implanted materials for electrical and mechanical integrity, using electrical testing and/or scanning electron microscopy, and of tissue reactions at the sites of implantation using routine histological methods.

## B. Designs for chronic recording intracortical microelectrodes.

The "map-pin" chronic microelectrode, originally developed in this laboratory, continues to be adapted and refined for new applications. The most recent refinement is for chronic recording of somatosensory receptive fields in the cat cuneate nucleus. To accommodate the unusual geometry and considerable movement at this site at the base of the skull, the electrodes have been modified to include a short length of twisted pair gold wire leads which terminates in a much heavier stranded stainless steel wire for eventual exit at a pedestal connector attached to the skull.

## C. Nerve cuff electrodes for recording from peripheral nerves.

The femoral nerve cuff electrode described in last year's Annual Report (two sets of tripolar electrodes plus infusion catheter) is now in regular use in chronic unit recording experiments from the cat L5 dorsal root ganglion and ventral roots. They have been excellent both for conduction velocity measurements and for differential nerve block. In the latter, the  $\gamma$ -innervation of quadriceps muscle spindles is blocked by injection of a low concentration xylocaine solution while the spindle primary afferent and  $\alpha$ -motoneuron activity remains intact and under continuous observation. Similar but simplified cuff electrodes are now also being used in the new cuneate nucleus project to record



field potentials from microstimulation of this nucleus via implanted electrodes and to electrically stimulate cuneate nucleus afferents from well controlled peripheral nerve fields.

D. Implantable tendon strain gauge.

The semiconductor tendon strain gauge developed in Laboratory of Neural Control several years ago continues to be implanted in cats as part of ongoing kinesiological studies. A number of modifications have been employed to adapt it to a variety of specialized tendon and implant site geometries encountered in a project intended to survey the normal function of all the muscles in the cat hindlimb which control or project across the ankle joint.

E. New treadmill for kinesiological studies using cats.

Construction was substantially completed on a novel treadmill design described in the previous Annual Report. An extensive survey and tests were undertaken to identify a method of powering the belt which is both electrically and acoustically quiet and continuously and smoothly variable in speed over a considerable range of torques and RPM's. Installation of the completed unit in the Neurokinesiology Facility is expected shortly.

F. Behavioral training apparatus for controlled postural movements by a standing cat.

The force plate system described in the previous Annual Report (four separate vertical force detectors for determining center of gravity changes in a standing cat) has been modified for a new behavioral paradigm. Cats will be trained to exert a specific amount of vertical force in one limb by using the output of a force plate to control the position of a moving feeding arm. The food reward only passes within reach of the cat's mouth when the force output is within a desired window.

G. Percutaneous connector and cable systems for chronic neurokinesiology studies.

The increasing complexity of normal activities which are being monitored in chronically implanted cats, plus increased numbers of data channels, have required continued development of connector and cable systems. The chief requirements include very low connector noise even during motion, minimal weight and torsion applied to the cat, ease and flexibility of wiring during surgery, and reliable but inexpensive fabrication for disposability. A set of custom printed circuit boards has been developed which allow use of a 40 pin ribbon cable connector which is a standard item in the computer industry. The system will incorporate the special 12 channel FET-hybrid preamplifier developed for the LNLCL for such applications.

H. Stimulus Isolator

A biphasic isolator has been designed and is now in routine use within the laboratory. It has two isolated inputs and can be used as two independent



constant current isolators or as a biphasic stimulus isolator by supplying a biphasic rectangular pulse to one of the inputs and tying the two sets of output jacks together. It has continuous control of calibrated constant current levels from 100 nanoamps to 10 milliamps.

#### I. Other laboratory instruments

The following list includes some of the special-purpose instruments designed, built and put into operation in the laboratory within the last year: 1) D.C. bridge amplifier system for detection of forces from implanted tendon strain gauge; 2) differential amplifier instrument with rectification and integration for detection of foot fall of a cat's paw on a moving treadmill; 3) LED display circuit for monitoring absolute depth measurements of glass micropipettes; 4) ramp hold generator for producing a pulse initiated ramp and hold voltage for producing smooth muscle stretches; 5) torque motor control amplifiers for position and velocity feedback control of monkey hand manipulandum; 6) control circuit and current/voltage monitoring for stimulus delivery to chronically implanted microelectrode; 7) a molded hand grip designed to be used in monkey training experiments in conjunction with a servo-control manipulandum and T.V. monitor feedback.

#### Major Findings:

"Map-pin" electrodes continue to provide excellent chronic recordings from single neural elements as well as field potentials in various regions of the nervous system. The modified version of the map-pin electrode now being used in the cuneate nucleus of cats appears to be working well at holding the same field potential for several days. Some problems have developed as far as implanting the electrodes in the forearm region for which micro-stimulation of the "map-pin" electrodes will be used for proper localization of the electrodes.

"Hair brush" electrode arrays have been routinely implanted in cat motor cortex and successfully recorded single unit activity for several months.

Although the modified version of the map-pin electrode showed continued success in recording from ventral roots in cat they are now used with less frequency depending on the experimental protocol because of their sometimes unwanted stability in recording from the same nerve fibers.

Tantalum and conventional "map-pin" electrodes were used successfully in long term microstimulation paradigms with the tantalum type electrodes showing more promise as stimulating electrodes. Some problems with the stability of the parylene insulation have been confirmed by visualization from scanning electron microscopy in which cracks appear. It is not clear yet whether this may be due to fabrication techniques employed or as a result of electrochemical forces during stimulation.

The newly designed stimulus isolator instrument has proven to provide lower noise outputs with no sacrifice in rise time or the ability to produce perfectly balanced constant current waveforms when used in conjunction with the biphasic pulse generator also designed in this laboratory.

Significance to Biomedical Research and the Program of the Institute:

The successful development of techniques for recording signals from the nervous system, and for delivering safe current levels and waveform parameters for neural stimulation, is essential to the success of ongoing experiments in LNLc. These newly developed techniques also benefit other laboratories involved in neurophysiological research and ultimately may have an impact in the development of prosthetic devices for the neurologically handicapped.

Proposed Course of Project:

Continued use of the "map-pin" electrode design for recording neurons in the superficial layers of the cerebral cortex is anticipated. Appropriate modifications to the electrode designs will continue to be made in order to adapt them to other recording situations, with special emphasis on array designs and implant procedures for chronic spinal cord studies and studies in the midbrain. Cats implanted with "map-pin" electrodes will be fitted with various EMG recording electrodes, strain and length gauges and nerve cuffs to permit detailed correlation of normal neural activity with various voluntary and reflex locomotory movement.

Refinements in manufacturing techniques for the tantalum and conventional stimulating "map-pin" electrodes will be instituted to improve the insulating qualities and to reduce the overall electrode size in order to improve selectivity.

Continued cooperation and communication with the Neural Prosthesis Program is anticipated. In addition, the information circular published annually by LNLc staff and supplied to investigators interested in chronic recording techniques will be continued. This informal publication now reaches about 100 investigators worldwide, at their request.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 01688-13 LNLC
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Cortical Mechanisms of Voluntary Motor Control		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Edward M. Schmidt, Ph.D. Other: Martin J. Bak George M. Dold Lloyd L. Glenn, Ph.D. Michael E. Gordon Frederick T. Hambrecht, M.D.  Joan S. McIntosh Claude I. Palmer, Ph.D. Margaretta Ringqvist, M.D.	Biological Engineer Electronics Engineer Engineering Technician Guest Worker Engineering Technician Head, Neuroprosthesis Program Physiologist Visiting Fellow Visiting Associate	LNLC NINCDS LNLC NINCDS LNLC NINCDS LNLC NINCDS LNLC NINCDS FNP NINCDS  LNLC NINCDS LNLC NINCDS LNLC NINCDS
COOPERATING UNITS (if any)  Fundamental Neurosciences Program, NINCDS		
LAB/BRANCH Laboratory of Neural Control		
SECTION		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2.8	PROFESSIONAL: 1.5	OTHER: 1.3
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINDRS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
<p>             This project is designed to investigate the size and spatial distribution of cortical "colonies" that are associated with individual muscles or closely related groups of muscles, as well as the activity of neurons in such colonies in the <u>motor cortex</u> during defined voluntary motor behaviors. <u>Intracortical microstimulation</u> (ICMS) is used to map regions that produce excitation or inhibition of particular muscles or muscle groups, and the resultant cortical maps are compared with these for synergist or antagonist muscle groups. <u>Cortical cell discharge patterns</u> during normal movements are evaluated with respect to the excitation or inhibition of muscle activity that is produced by ICMS. A study of the histochemistry of monkey forearm muscles is being conducted. Intracortical capacitor stimulating electrodes are being evaluated for efficacy, stability and safety for chronic implantation.           </p>		

## Project Description:

**Objectives:** The major goals of this project are: 1) to examine the spatial organization of motor cortex outflow to particular muscles or muscle groups during free or goal directed movement in awake animals; 2) to determine whether the firing patterns of small sets of cortical neurons contain sufficient information to specify the details of motor performance; 3) to examine the activity of closely located cortical neurons recorded from the same electrode to determine to what extent the sensori-motor cortex is organized into "colonies" of cells with similar function; 4) to determine if pyramidal tract (PT) neurons classed according to their sensory input respond in a similar manner under a variety of movements; 5) to determine the spatial distribution of fiber types in monkey forearm muscles with histochemical staining methods and compare these with muscle usage and cortical cell firing patterns during motor performance; and 6) to determine the safest, most effective stimulation parameters and electrodes for intracortical microstimulation.

## Methods Employed:

Monkeys are initially trained to produce defined movements of the wrist and forearm. In a current paradigm, the monkey manipulates a handle that positions a cursor on a T.V. screen and, to receive a liquid reward, is required to match the position of this cursor for a prescribed length of time to a computer-generated target zone also displayed on the T.V. screen. A computer controls the entire experiment, including target and response displays, generation of random on-target hold times, and delivery of rewards. When the animal is well trained, a chamber to support the microelectrode drive is implanted over the hand-arm area of the motor cortex and a pyramidal tract stimulating electrode, EMG electrodes, and tendon strain gauge are implanted. After recovery from surgery, the monkey is retrained to move the handle between two targets under a stimulated spring load condition. The load is zero in a neutral wrist position and increases linearly in either direction to oppose movements. Task correlated cells are sought with a moveable microelectrode. When a cell of interest is isolated, stimulation is applied through the pyramidal tract electrode for cell classification. During the movement task, data is collected about movement parameters, EMGs, and activity of the cortical neuron. Sufficient cell firing data is collected to utilize spike triggered averaging to determine if the cell under investigation facilitates or suppresses the EMG activity of the implanted forearm muscles. Microstimulation is applied through the cortical electrode as single pulses at 12.5 Hz during the task, or as trains of 17 pulses at 400 Hz at specific times during the task to determine which muscles respond from the same cortical site. Cortical maps of muscle representation are constructed for the two types of microstimulation to determine if high frequency trains produce an enlarged map.

Intracortical capacitor stimulating electrodes are being developed under the neuroprosthesis contracts program by Giner, Inc. and are chronically implanted in the motor cortex for evaluation.

Studies of muscle histochemistry in monkeys are performed on muscles removed from animals sacrificed in the normal course of other experiments. Forearm muscles are removed in toto and rapidly frozen in isopentane cooled with liquid



nitrogen. 10  $\mu\text{m}$  frozen sections are cut from selected portions of the muscles and stained for ATPase activity after alkaline and acidic preincubation ATPase, and for NADH-tetrazolium reductase activity type I, type IIA and type IIB fibers are identified by standard criteria.

### Major Findings:

Intracortical microstimulation (ICMS) through a microelectrode while recording from chronically implanted EMG electrodes provides a means of determining how muscles are affected by stimulation of specific, highly-localized cortical sites. Both proximal and distal muscles can be activated by trains of pulses at current levels below 20  $\mu\text{A}$ . The effects in a given muscle can be generated by ICMS in widely separated areas, suggesting that cortical "colonies" related to that muscle can be very extensive and perhaps discontinuous. Excitatory or inhibitory EMG responses to ICMS can occur within 11 ms after the start of the stimulus train and end 11 ms after the end of the train. The prompt termination after the end of the stimulus train suggests that the onset and offset latencies are due mainly to conduction and synaptic delays rather than to reverberating cortical circuits that might be expected to remain active after the end of stimulation. EMG patterns produced by ICMS have ranged from inhibition or excitation of a single muscle to simultaneous inhibition and excitation of synergists or antagonists. Areas that produce inhibition of a given muscle have been observed as close as 100  $\mu\text{m}$  to areas producing excitation of the same muscle. Cortical sites that produce inhibition in a single muscle tend to surround those areas where ICMS produces excitation in the same muscle. Pure inhibition with ICMS has been observed predominantly in forearm flexor muscles.

Task-related activity in individual cortical neurons has been evaluated at sites where either excitation or inhibition of forearm muscles was produced with ICMS. Cells that exhibited consistent alterations in firing patterns before and/or during specific muscle activity are referred to as correlated with the muscle(s). The majority of cells whose firing patterns were correlated with increased flexor muscle activity during the task were recorded at sites where ICMS produced flexor excitation. The majority of cells whose firing patterns were correlated with increased extensor muscle activity during the task were recorded at sites where ICMS produced extensor excitation and/or flexor inhibition. At sites where only flexor inhibition was produced by ICMS, the firing patterns of the majority of cells were correlated with extensor muscle activity. Cells whose firing patterns were correlated with flexor muscle activity could be involved in the excitation of flexor muscles and/or inhibition of extensor muscles. Likewise cells whose firing patterns were correlated with extensor muscle activity could be involved in excitation of extensor muscles and/or inhibition of flexor muscles. The fact that only flexor inhibition was produced with ICMS at specific sites where the majority of cells were correlated with extensor muscle activity suggests that these cells might be involved in active inhibition of flexor muscles. If some cortical cells produce only inhibition of muscle activity, then cells recorded at sites where ICMS produces inhibition should be active when the target muscle is inhibited. Likewise at sites where ICMS produces excitation, the cells recorded should be active when the target muscles are active. The majority of cells follow this expected ICMS pattern but a substantial number do not, suggesting that the cortical outflow to



different muscles overlaps in space and that the functional effect of individual cortical cells may be complicated by their connectivity in the spinal cord segments to which they are assumed to project.

In superficial monkey forearm muscles, we have found an essentially constant mosaic of type I and type II fibers throughout the muscle in all but flexor carpi ulnaris (FCU). FCU is a bipennate muscle with distinctly different proportions of type I and type II fibers on either side of a central tendon. The two parts of FCU originate as two separate heads. The lateral head, which is predominately type II, originates from the ulna while the humeral head is predominately type I. A similar situation has been found in the cat except that the two parts of FCU are separate muscles with a common tendon.

In order to evaluate newly fabricated miniature capacitor electrodes, originating from an earlier LNL design, an array of nine tantalum pentoxide capacitor electrodes and two iridium electrodes were chronically implanted in the motor cortex of a monkey for approximately two months. Threshold currents for muscle activation with the capacitor electrodes remained relatively stable for many days. However, the electrodes tended to migrate slowly in the cortex such that stimulation of the same electrode elicited movement of different muscles during the course of the implant. The voltage transients across the capacitor electrodes during stimulation remained relatively stable in contrast to conventional iridium metal electrodes, which exhibited nonlinear transients suggesting gassing when they had not been stimulated for a number of days. High current stimulation was evaluated with one of the capacitor electrodes at the end of the experiment to obtain a physiological measure of safety. Trains of stimuli up to seven times threshold current were applied for periods of 1.5 minutes with no change in threshold current suggesting that stimulation with these electrodes produced little or no local tissue damage. Scanning electron micrographs of the electrodes after removal from the brain showed that there was no tissue ingrowth into the pores of the electrode and that the tantalum pentoxide surface withstood the hostile environment of the body.

#### Proposed Course of Project:

Work will continue on the behavior of cells in the motor cortex, their relationship to movement parameters and the EMG responses produced by microstimulation at the recording site. Attention will concentrate on cortical cells related to activity in the markedly heterogeneous FCU, especially to the question whether the two heads are used differently in tasks such as flexion vs. ulnar deviation and also fast versus slow movements. Cortical maps of locations where microstimulation excites or inhibits the two parts of FCU will be constructed to determine if separate regions of cortex control the two compartments.

A collaborative program with Dr. A.J. Berman of the Bronx V.A. Hospital has been initiated to study cortical cell firing patterns in monkeys with unilateral or bilateral dorsal root section in the arm cord. All studies will employ animals that Dr. Berman has rhizotomized and on which behavioral studies were conducted during recovery of movement. The initial studies will record from cortical cells in normal and deafferented cortex of monkeys with unilateral rhizotomies to determine the effect on firing patterns during movements of sensory feedback removal. Another task to be employed with these animals is to

produce operant conditioning of the firing patterns of cortical cells in the deafferented cortex. One hypothesis that will be tested is that the operant conditioning of cortical neurons requires sensory feedback.

Further studies will be conducted with chronically implanted intracortical capacitor electrodes to evaluate their efficacy and safety. A new fabrication technique is under investigation that will produce a smaller capacitor electrode that will allow stimulation of smaller cortical volumes.

#### Significance to Biomedical Research and the Program of the Institute:

The motor cortex and possibly the supplementary motor area are intimately involved in the production of distal, exploratory movements with hands and digits in primates. These functions are disturbed by stroke in many human patients. The mechanisms of compensation for motor deficits caused by cerebral lesions are unknown but information about normal cortical mechanisms and their stability (or instability) with time is important to increase our basic understanding which then can be applied to lesion problems. Studies on the dynamic activity of cortical neurons and their relationship to movement, along with the spatial organization of neurons related to a specific muscle or movement will provide this basic information. With our newer microstimulation and microelectrode recording techniques we can better attack the question of whether the cortex contains a representation of muscles or of movements. Because all movements involve the contraction (or relaxation) of one or more muscles, the basic representation must be in terms of muscles but the large spatial extent of cortical "colonies" to a specific muscle and the overlap of the colonies that project to different muscles tend to indicate that any given cortical region may be involved in specifying combinations of muscles that produce a movement.

Utilizing EMG recordings along with intracortical microstimulation at specific times during a trained movement task allows evaluation of the efficacy of particular stimulation parameters. Comparisons can be made between intracortical metal and capacitor electrodes as to efficacy and long term safety. Determination of the safest stimulation values and electrodes will be directly applicable to neuroprosthesis applications. The newly developed intracortical capacitor electrodes will provide a means for determining if a visual prosthesis can be developed.

The ability of the chronically implanted "map-pin" electrodes to record the activity of the same cortical neurons for many days, weeks and even months should prove useful for evaluating drugs. This work may also have significance for the development of cortically-controlled prosthetic devices, although the electrodes are not yet satisfactory for obtaining prosthetic control signals where recordings for a number of years are required.

#### Publications:

Palmer, C., Schmidt, E.M. and McIntosh, J.S.: Corticospinal and corticorubral projections from the supplementary motor area in the monkey. Brain Res. 209:305-314, 1981.

Schmidt, E.M. and Thomas, J.S.: Motor unit recruitment order: Modification under volitional control. Prog. Clin. Neurophysiol. 9:145-148, 1981.

Schmidt, E.M.: Single neuron recording from motor cortex as a possible source of signals for control of external devices. Annals of Biomed. Eng. (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02079-08 LNLC
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Models of Neural Interactions		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: William B. Marks, Ph.D. Other: Lawrence D. Abraham, Ed.D. Joaquin A. Hoffer, Ph.D. Gerald E. Loeb, M.D. Naotoshi Sugano, Ph.D. Michael M. Merzenich, Ph.D.  Mark White, Ph.D.	Research Physiologist LNLC NINCDS Guest Worker LNLC NINCDS Senior Staff Fellow LNLC NINCDS Medical Officer (Res.) LNLC NINCDS Visiting Fellow LNLC NINCDS Professor, Dept. Physiology, U. Calif. at San Francisco Adjunct Research Professor, Dept Electrical Engineering, U. Calif. at San Francisco	
COOPERATING UNITS (if any)  Coleman Memorial Laboratory, Dept. Otolaryngology, U. of California at San Francisco School of Medicine		
LAB/BRANCH Laboratory of Neural Control		
SECTION		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.1	PROFESSIONAL: 1.0	OTHER: 0.1
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SUMMARY OF WORK (200 words or less - underline keywords)  This project is concerned with the detection of patterns of simultaneous activity among groups of neurons and muscles, with describing these patterns mathematically, and with the underlying principles of nervous organization that these patterns may exemplify.		

## Project Description:

**Objectives:** This project continues to be concerned with developing conceptual and technical approaches for detecting and quantitating patterns of interrelated activity among groups of neurons and muscles during normal use and for processing neural and kinesiological data to reveal underlying patterns. Examples include simultaneous multi-channel data streams that include electrical activity of neurons in the central and/or peripheral nervous system, electrical and mechanical activity of individual muscles, and whole animal movements. A second aspect involves theoretical and experimental studies of information processing in ensembles of neurons.

## Methods Employed

The experimental data base for this project originates largely from studies of movement control described in other sections of this report (see projects Z01 NS 02080-08 LNLc and Z01 NS 01688-13 LNLc). This year has required an intensive effort to design a flexible general approach to the problem of reducing multichannel data streams and to build a library of computational modules and of complete programs for the PDP-11 laboratory computers in use in LNLc. Many of these have been written in the new computer language 'C', although other languages (Fortran, APL, Pascal and Basic) are also used as appropriate. These have included complete programs for analysing movements (joint angles, limb trajectories, etc.) using a joystick-controlled cursor superimposed on videotaped records of moving animals and for acquiring multichannel data rapidly around the time of a stimulus, followed by detailed processing and display.

## Major Findings

### A. Development of Data Processing Techniques.

It was necessary to develop a method to permit comparison of response patterns of neurons in the sensorimotor cortex of intact cats during normal stepping and dropping from heights with responses to stimulation of nerve bundles in the foreleg during the same types of stepping and dropping movements. The problem is complicated by the fact that no two stepping cycles are exactly the same duration, nor are the discharge patterns of neurons ever exactly alike. Thus, a computational approach was required that enabled: 1. automated search or analysis epochs with similar characteristics (e.g., step durations, phasing of stimuli, etc.); and 2. normalization of temporal features to permit tests of repeatability of responses. Limits of acceptability had to be imposed and the final computational approach required considerable interaction with the experimenter.

It was often observed that the 'sensory' responsiveness of cortical neurons increased at particular periods during the step (or dropping) cycle, when the 'motor' activity (i.e., that associated with the movement in the absence of nerve stimulation) was decreasing, or vice versa. This dissociation between responsiveness to input and spontaneous activity was similar for walking and dropping. This finding is compatible with the existence of "gating" of sensory



information to neurons in the sensorimotor cortex during performance of movements in which the neurons under observation are presumed to play a role.

A basically similar analysis problem was faced in dealing with data from the hindlimb of walking cats, in which electromyographic activity in several muscles are simultaneously monitored in the presence and absence of electrical stimuli delivered to peripheral nerves at various phases of individual step cycles. The analysis approach adopted was to compress the responses and normal activity during hundreds of steps into one highly processed computer display, from which the patterns of reflex EMG responsiveness clearly emerge in relation to the phase of the step cycle. Although flexor muscles are generally responsive only during flexion and extensors only during extension, responsiveness is not always coincident with the phasing of normal activity, and the responses of natural agonists may be quite different. At present the growing mass of such data increasingly emphasizes the uniqueness of each muscle, within which it is possible to look for new patterns of synergy.

Recent progress in obtaining well-characterized muscle spindle unit activity from the quadriceps and sartorius muscles of the cat hindlimb has led us to attempt to model the factors which contribute to such spindle afferent activity. In particular, this preparation provides continuous multichannel data streams that also include parent muscle length, EMG, and tension output during normal activities. Selective blockade of fusimotor action on the spindle by infusion of dilute xylocaine around the femoral nerve permits controlled modulation of fusimotor bias during normal stepping movements. The resulting data is now being processed to develop quantitative descriptions of the extrinsic and intrinsic mechanical factors which influence spindle afferent activity. In so doing, account can be taken of the known behavior of cat muscle spindles in anesthetized animals or under in vitro conditions. Various model formulations of spindle behavior are being compared with experimental data obtained under conditions of normal and reduced fusimotor bias. This should lead to a clearer picture of the activity and functional role of the fusimotor system, which because of technical limitations, can be achieved only in this deductive manner.

## B. Independent Source Theory.

In past years, we have developed a theoretical formulation by which the correlated activity in ensembles of active elements can be described in terms of the independent processes driving such activity. A number of parallels have been observed between the quantitative process of resolving correlated signals into their independent components and the qualitative process of explaining patterns of observations in terms of underlying hypothetical "objects". Complex systems like nervous systems are usually modeled and thought about in terms of objects ("black boxes") which interact according to unknown rules. Some objects are observable, but the properties and connections of others must be inferred from patterns of interactions. Most studies of nervous systems consist of efforts to identify all of the aspects of the data that can be accounted for by the properties of known objects (electrical interference, characteristics of the equipment, and previously discovered components of the system under study). Then, as in the quantitative situation, it is often noticed that the still unexplained observations contain a pattern, as though influences from a

previously undetected aspect of the system were fanning out to produce correlations in the observations. This insight constitutes a new hypothesis to guide further experimentation. The continued development of independent source theory is designed to test the notion that the unknown properties of the elements of the system, and the rules governing their interactions, can emerge in this way from systematic examination of activity patterns in ensembles of system elements (e.g., neurons). The applications described above can be considered as special cases of this general approach.

### C. Information Processing in the Auditory System.

The collaborative study initiated last year with Dr. Michael M. Merzenich, Director of the Coleman Memorial Laboratory, Dept. of Otolaryngology, U.C.S.F., has been actively pursued this year on two fronts. First, a specific model of the processing of auditory signals has been sought which will account for the psychophysics of normal and prosthetic auditory sensations and which will make specific, experimentally-testable predictions about the anatomy and physiology of auditory brain stem nuclei. Second, acute neurophysiological preparations and techniques are being developed which will permit direct tests of the model's predictions and will generate data to guide the refinement of the model. This project is directed toward the development of a comprehensive theory of pitch detection for the band of frequencies which produce phase-locked discharge in first and second order cochlear afferents (approximately 500 to 5000 Hz, the so-called "speech frequencies"). This model is intended to overcome deficiencies in the existing place-pitch and periodicity-pitch models which have come to light as a result of psychophysical testing of patients recently implanted with multi-channel intracochlear stimulation electrodes as an auditory prosthesis.

The theory described in last year's Annual Report has been considerably extended and refined. We now believe that the detection of both pitch and stereophonic sound localization (based on interaural timing information) are accomplished at a single stage in the medial superior olive (MSO). Computer simulations of the model have pointed out an unexpected feature which predicts the optimal sampling interval of the spatial-temporal cross-correlator which we believe exists in the MSO. This interval agrees quite closely with an anomaly which we have discovered in the tonotopic frequency map of the antero-ventral cochlear nucleus (AVCN, the major ascending input to the MSO).

A direct neurophysiological test of our Synchronicity Theory of Pitch is predicted by the model, namely that there is a non-linear multiplicative summation in the MSO of at least two inputs from adjacent areas of each AVCN. This interaction should be highly sensitive to time of input signal arrival. Theoretically, this could be tested by a three microelectrode experiment (all in closely related frequency areas of the MSO and AVCN) in which microstimulation of the AVCN provides a temporally and spatially controllable pair of input signals. Most of the past year has been spent in overcoming the formidable technical problems arising from the inaccessibility of both target structures and the previously experienced difficulty of recording usable signals from identified MSO neurons. While these problems have been overcome, initial experiments have revealed another technical problem regarding interaction between the stimuli, which remains to be overcome.

Significance to Biomedical Research and the Program of the Institute:

The extraction of information about the operation of nervous systems from records of activity patterns in a subset of neural elements is a central problem in neurophysiology. New developments in LNLN provide the opportunity to examine patterns of activity in a number of neurons and muscles simultaneously during normal activity, using a variety of sensors and monitoring devices. Reduction of such data streams is complicated by the fact that movements are almost never completely stereotyped or absolutely repeatable. In addition, the ultimate object of data reduction is to seek to detect and display information embedded rather deeply in the data. The development of general approaches to this problem, and of specific solutions to specific problems, are important not only to ongoing research in LNLN but also to research work on many types of neural systems.

Proposed Course of Project:

The next year should be mainly devoted to improving and using the computer-based data analysis packages, applying them to ongoing research problems in awake, behaving animals. For example, it should become possible to enlarge the present model that predicts spindle firing to include an inferred signal from gamma efferents. Existing data from previous experiments and new data from ongoing experiments will be processed by computer programs for fitting proposed models of spindle control. Goodness of fit will be evaluated and compared for various models. It is anticipated that new models will be suggested by this process and similarly evaluated. We will continue to study changes in reflex responses in motoneurons during the stepping cycle, cataloging such changes for a number of muscles and stimulated nerves for comparison with data from reduced preparations. It should be possible to feed kinesiological records directly into the computer with a new joystick controlled video analyser. Finally it will become possible to begin to fit the activity of a number of muscles to a small number of hypothetical underlying variables, first the phase of the stepping cycle, and then correctional patterns superimposed on the basic pattern. The series of collaborative neurophysiological experiments currently underway on auditory processing in the MSO and AVCN will be completed and the results incorporated into the currently developing theory of Synchronicity Pitch perception. A new series of auditory prosthesis patients with improved electrode geometry is scheduled for implantation in San Francisco at the end of the current year and they will be tested psychophysically to provide further data regarding the percepts generated by controlled electrical activation of the auditory pathways.

Publications:

Loeb, G.E. and Marks, W.B. Epistemology and heuristics in neural network research. Behav. and Brain Sci. 3:556-557, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02080-08 LNLc																																																							
PERIOD COVERED October 1, 1980 to September 30, 1981																																																									
TITLE OF PROJECT (80 characters or less)  Neuron Activity During Locomotion																																																									
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<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 40%;">Gerald E. Loeb, M.D.</td> <td style="width: 20%;">Medical Officer (Res.)</td> <td style="width: 10%;">LNLc</td> <td style="width: 10%;">NINcDS</td> </tr> <tr> <td>Other:</td> <td>Lawrence D. Abraham</td> <td>Guest Worker</td> <td>LNLc</td> <td>NINcDS</td> </tr> <tr> <td></td> <td>Martin J. Bak</td> <td>Electronics Engineer</td> <td>LNLc</td> <td>NINcDS</td> </tr> <tr> <td></td> <td>Robert E. Burke, M.D.</td> <td>Chief</td> <td>LNLc</td> <td>NINcDS</td> </tr> <tr> <td></td> <td>Joaquin A. Hoffer, Ph.D.</td> <td>Senior Staff Fellow</td> <td>LNLc</td> <td>NINcDS</td> </tr> <tr> <td></td> <td>William B. Marks, Ph.D.</td> <td>Research Physiologist</td> <td>LNLc</td> <td>NINcDS</td> </tr> <tr> <td></td> <td>Randall J. Nelson, Ph.D.</td> <td>Staff Fellow</td> <td>LNP</td> <td>NIMH</td> </tr> <tr> <td></td> <td>Claude I. Palmer, Ph.D.</td> <td>Visiting Fellow</td> <td>LNLc</td> <td>NINcDS</td> </tr> <tr> <td></td> <td>Martin J. Pinter, Ph.D.</td> <td>Guest Worker</td> <td>LNLc</td> <td>NINcDS</td> </tr> <tr> <td></td> <td>Andrew Rindos</td> <td>Guest Worker</td> <td>LNLc</td> <td>NINcDS</td> </tr> <tr> <td></td> <td>Naotoshi Sugano, Ph.D.</td> <td>Visiting Fellow</td> <td>LNLc</td> <td>NINcDS</td> </tr> </table>			PI:	Gerald E. Loeb, M.D.	Medical Officer (Res.)	LNLc	NINcDS	Other:	Lawrence D. Abraham	Guest Worker	LNLc	NINcDS		Martin J. Bak	Electronics Engineer	LNLc	NINcDS		Robert E. Burke, M.D.	Chief	LNLc	NINcDS		Joaquin A. Hoffer, Ph.D.	Senior Staff Fellow	LNLc	NINcDS		William B. Marks, Ph.D.	Research Physiologist	LNLc	NINcDS		Randall J. Nelson, Ph.D.	Staff Fellow	LNP	NIMH		Claude I. Palmer, Ph.D.	Visiting Fellow	LNLc	NINcDS		Martin J. Pinter, Ph.D.	Guest Worker	LNLc	NINcDS		Andrew Rindos	Guest Worker	LNLc	NINcDS		Naotoshi Sugano, Ph.D.	Visiting Fellow	LNLc	NINcDS
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SUMMARY OF WORK (200 words or less - underline keywords) A variety of new techniques are being used to monitor the <u>afferent</u> and <u>efferent</u> <u>neural activity</u> in the <u>spinal cord</u> of intact cats during normal and perturbed <u>locomotion</u> . Flexible wire electrodes in the lumbar <u>dorsal root ganglia</u> (DRG) and <u>ventral roots</u> record stable, identifiable unit activity which is correlated with kinesiological data from chronically implanted gauges of muscle force, length, and EMG activity developed for this project. Neurons are characterized by conduction velocity, anatomical origin, and modality using <u>spike-triggered averaging</u> of EMG signals and neurograms obtained from specially designed <u>nerve cuff electrodes</u> implanted around peripheral nerves. The <u>reflex effects</u> of various electrical stimuli to motor and cutaneous nerves are systematically examined as they vary through the step cycle. The normal functional use of the various hindlimb muscles is being surveyed during a variety of normal behaviors in an attempt to correlate these patterns with their anatomical specializations regarding muscle fiber type and orientation and proprioceptive feedback. A new study has been initiated in which the activity in the <u>cuneate nucleus</u> , the first ascending relay for sensory information from the cat forelimb, will be chronically recorded to study plasticity following peripheral nerve ligation.																																																									



## Project Description:

**Objectives:** The major goal of this project is to examine directly the roles of spinal neurons and primary afferent fibers in normal movement which up till now have only been inferred from paralyzed or decerebrate acute preparations. The principal current emphasis is on natural patterns of afferent and efferent activity in the spinal cord and the role of afferents as part of a system of control mechanisms. Spinal reflexes elicited by both cutaneous and proprioceptive afferent activity are now known to have a profound effect on locomotory patterns and the effects of such perturbations on movement and neural activity are also being studied.

Two other projects in this laboratory continue to produce data from acute experiments concerning anatomical specializations of various hindlimb muscles. This has included their muscle fiber histochemistry and mechanical properties and their inter-relations with each other via local and distant cutaneous and proprioceptive circuits. An ongoing collaboration has begun to find correlations between such specializations and the functional uses and synergies of these muscles during a variety of normal behaviors. Conversely, unusual functional patterns discovered in this work are being used to suggest productive areas of inquiry for anatomical and spinal cord pathway studies.

Recent evidence indicates that there is complex and widespread reorganization of the somatotopic map in areas 1 and 3b of primary somatosensory cortex following localized loss of input from a peripheral nerve field. The actual site and mechanism of this plasticity is unknown. The chronic recording technique in the cuneate nucleus will allow us to study the first ascending relay before and after peripheral nerve ligations. Initial feasibility studies in cats have employed chronic floating ("map-pin") microelectrodes in the cuneate nucleus (first ascending relay) and chronic nerve cuff electrodes in forelimb (ulnar, median and radial nerves) for recording and stimulation.

## Methods Employed:

1. The present method for obtaining stable afferent unit records during normal walking consists of inserting insulated 50  $\mu$ m diameter wires into the dorsal root ganglion (DRG) via a small laminotomy. The cut ends of the wire constitute the recording surface and the only fixation is by a flexible Silastic carrying sleeve sutured to the dorsal spinous process.

2. A similar technique has been successfully employed in the ventral roots of the 5th lumbar spinal segment. Efferent unit activity in the axons of motor neurons is identified as such by spike-triggered averaging of the records obtained from a cuff electrode chronically implanted around the femoral nerve and from EMG electrodes designed to sample each of the five anterior thigh muscles to which this nerve projects (see also the report of project Z01 NS 01687-13 LNLCL).

3. Kinesiological measurements include continuous read-out of positional signals at ankle, knee, and hip joints via implanted length gauges, of the force generated by individual muscles via chronically implanted tendon strain gauges, and of overall movement patterns by videotape gait analysis.

4. Techniques have been developed to implant large numbers of bipolar recording and stimulating electrodes at a variety of sites in both hindlimbs of



a freely walking cat, permitting analysis of EMG reflexes elicitable by electrical stimulation of various afferent classes during stepping.

5. A new technique has been developed which allows the selective infusion of nerve blocking agents such as xylocaine around a given peripheral nerve while the animal is behaving normally. Since axons of differing size can be selectively and reversibly blocked pharmacologically, we are now able to examine the effects of sudden losses of afferent information or fusimotor control on the activity of the remaining neurons during the performance of voluntary movements.

6. The technique of generating controlled decerebrate locomotion by stimulating the midbrain locomotory region has been successfully combined with recordings from single spinal units previously observed during normal locomotion.

7. Chronic recording techniques previously employed for cortical and spinal cord sites are being evaluated and modified for the cuneate nucleus. Nerve cuffs have been developed for implantation on forelimb nerves (median, radial, and ulnar) where they will be used for anterograde stimulation and recording of antidromic activity from microstimulation of the cuneate microelectrodes.

8. An extensive hardware and software system has been devised to facilitate interactive reduction and analysis of neurophysiological and kinesiological data produced by the various projects using the neurokinesiology facility. Some of this is described under other projects of this Annual Report.

#### Major Findings:

Work to date in this and other laboratories regarding the normally occurring activity of muscle spindle afferents has demonstrated a number of very different patterns, each of which would tend to support only some of the conflicting theories of muscle spindle function. The central question is whether these afferents are, in fact, accurate sensors of muscle length and/or velocity of length changes, or whether their complex and powerful intrafusal fibers are used dynamically to create afferent patterns which subserve some other role (e.g. trajectory error detection). One possibility is that the variety of experimental observations may be attributed to the different muscles and tasks which different investigators have chosen to study. This would then imply that spindles do subserve multiple roles, a situation consistent with the high degree of intrafusal control arising from the gamma (and possibly beta) motoneurons, a prominent feature of most mammalian motor neuron pools.

The functional de-efferentation of spindles during normal behavior by femoral nerve perfusion with xylocaine has confirmed the high degree of fusimotor activity that is present and modulated during normal activity such as walking. In some cases, it has been possible to fractionate more than one distinct fusimotor influence on a single spindle afferent by successive increments of nerve block. This has made possible the current attempts to generate quantitative models of spindle function to describe and explain the considerable heterogeneity in activity observed to date for different spindle afferents.

The increasing data base for motoneuron discharge patterns during normal locomotion is now sufficient to make additional statements regarding their normal recruitment. In particular, it now appears that, in normal walking, in contrast to motoneurons rarely, if at all, fire with initial doublet bursts of activity when recruited. Rather, each motoneuron is recruited in an orderly and reproducible manner as the overall level of effort of the muscle reaches some

fixed level (based on gross EMG integration), with the firing rate smoothly modulated between about 10 to 40 pps dependent on the overall level of muscle activity. This is somewhat disappointing in view of the literature which points out the advantages in force rise-time and force-time integral (momentum) resulting from initial doublet activity. It remains to be seen whether doublet activation has a role in other motor tasks, where such properties might be more valuable to the animal.

The detailed examination recently begun of the kinesiology and kinematics of the cat hindlimb musculature has produced a variety of interesting findings. First, we now have a much better understanding of the high degree of anatomical specialization which exists among nominally synergistic muscles around the ankle joint. For example, the muscle fiber type distribution and orientation in the tibialis posterior muscle suit it to generating high forces for virtually isometric lengths; its insertion permits such forces to contribute to ankle extension only at highly extended angles. It was, therefore, gratifying to find that the muscle works in exactly this manner during walking and trotting; it, alone, generates the final push-off thrust at the end of the stance phase while the other ankle extensors, shortened past their optimal lengths, fall silent. Another unusual anatomical arrangement can be found in the plantaris and flexor digitorum brevis muscles, which are connected in series with each other with a common intervening tendon. As might be expected, these muscles are usually turned on simultaneously, although not always proportionately. It is possible that this pair functions as a variable stiffness spring which can be set to a variety of resting length positions. This study has also followed up on previously reported work from this laboratory regarding the specialized uses of the flexor hallucis longus and flexor digitorum longus muscles, which have identical anatomical action but very different physiological recruitment. Previous findings have been confirmed and extended to include differences in their patterns of reflex response following cutaneous stimulation during walking. Simultaneous recordings from up to 11 separate distal hindlimb muscles has permitted us to develop a comprehensive picture of their detailed phasing and work output during complex, rapid activities such as paw shaking and scratching. The simultaneous monitoring of EMG, muscle length, and tendon tension in normally functioning muscles continues to point out the highly complex interplay of these factors in determining the mechanical output of active muscles.

#### Significance to Biomedical Research and the Program of the Institute:

Much current work on the study of mammalian locomotion has been concentrated on the cat hindlimb, where considerable knowledge is already available concerning the physiological and anatomical properties of the muscles, motor neurons, afferents, and spinal reflexes. However, the details of the functioning of this system during normal locomotion under cerebral control can at present only be inferred, giving rise to a number of competing control theory hypotheses. The new methods employed in this project should provide data needed for testing such hypotheses and formulating new ones. An understanding of the normal control of movement is essential to understanding a number of degenerative diseases of the spinal cord (e.g. ALS, transverse myelitis, etc.) that affect locomotion. A longer range application of the techniques using chronic transducers and

afferent monitoring is in the field of functional neuromuscular stimulation (FNS). Sophisticated devices designed to restore motor function by bypassing CNS lesions (e.g. in paraplegics) will probably require some form of closed loop servo-control utilizing transducers of muscle length and tension and of skin pressure. If it proves possible to obtain stable afferent activity rather than using implanted artificial transducer signals over long periods of time, afferent recording electrodes could improve the function and simplify the design and implantation of complete FNS systems.

### Proposed Course of Project:

A major objective of this project is the determination of the functional spinal organization underlying the generation and regulation of locomotion. The most useful sources of data continue to be the single unit activity of muscle afferents (particularly spindle endings), the EMG and force outputs of selected muscles, and unitary activity from identified spinal cord motoneurons. The experimental paradigms to be investigated include the various "voluntary" modes of locomotion, the effects of singular perturbations such as electrical and mechanical stimuli during locomotion and the execution of normal stereotyped motor programs other than locomotion, such as scratching, paw shaking, jumping, and postural shifts during standing. Quantitative modeling of the factors contributing to observed neural activity will be a major goal.

The ongoing collaboration and exchange of personnel between the neurokinesiology projects described here and the acute spinal cord and muscle research projects in the LNLc ( $\alpha$ 01686 and  $\alpha$ 02160) promises to be a productive source of leads for concentrated attacks using both approaches.

Additional work is needed to obtain an adequate number of cuneate electrodes with fields distributed among the desired peripheral nerves. Future implantations will include attempts to microstimulate through the electrodes while recording from the newly implanted peripheral nerve cuffs to guide electrode positioning. When a reliable cuneate preparation has been achieved, some pilot experiments looking at peripheral nerve ligations in cats will be pursued. Ultimately, the project will duplicate the ligation experiments previously done for monkey somatosensory cortical mapping in an attempt to show how much reorganization appears to occur at this first synaptic relay.

A long-term technological goal is to determine the limits of recording device stability and tissue compatibility in both time and numbers of information channels with a view to assessing the feasibility of deriving somatosensory information for the feedback control of Functional Neuromuscular Stimulation Prostheses.

### Publications:

Duysens, J. and Loeb, G.E. Modulation of ipsi- and contralateral reflex responses in unrestrained walking cats. J. Neurophysiol. 44:1024-1037, 1980.

Duysens, J., Loeb, G.E., and Weston, B.J. Crossed flexor reflex responses and their reversal in freely walking cats. Brain Res. 197:538-542, 1980.

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Hoffer, J.A., Loeb, G.E. and Pratt, C.A. Single unit conduction velocities from averaged nerve cuff electrode records in freely moving cats. J. Neurosci. Methods (In press).

Hoffer, J.A., O'Donovan, M.J., Pratt, C.A. and Loeb, G.E. Discharge patterns of hindlimb motoneurons during normal cat locomotion. Science (In press).

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Loeb, G.E. and Hoffer, J.A. Muscle spindle function during normal and perturbed locomotion in cats. In Taylor, A. and Prochazka, A. (Eds.) Muscle Receptors and Movement London: MacMillan, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> <b>INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 NS 02160-07 LNLC														
PERIOD COVERED October 1, 1980 to September 30, 1981																
TITLE OF PROJECT (80 characters or less)  Intrinsic Properties of Motor Units																
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																
<table style="width: 100%; border: none;"> <tr> <td style="width: 45%;">PI: Robert E. Burke, M.D.</td> <td style="width: 45%;">Chief LNLC NINCDS</td> </tr> <tr> <td>Other: James W. Fleshman, Ph.D.</td> <td>Guest Worker LNLC NINCDS</td> </tr> <tr> <td>G.F. Gauthier, Ph.D.</td> <td>Prof. Anatomy, U. Mass. Med. Schl.</td> </tr> <tr> <td>Loyd L. Glenn, Ph.D.</td> <td>Guest Worker LNLC NINCDS</td> </tr> <tr> <td>Aharony Lev Tov, Ph.D.</td> <td>Visiting Fellow LNLC NINCDS</td> </tr> <tr> <td>Richard F. Mayer, M.D.</td> <td>Dept. Neurology, U. Maryland</td> </tr> <tr> <td>James Toop, Ph.D.</td> <td>Dept. Neurology, U. Maryland</td> </tr> </table>			PI: Robert E. Burke, M.D.	Chief LNLC NINCDS	Other: James W. Fleshman, Ph.D.	Guest Worker LNLC NINCDS	G.F. Gauthier, Ph.D.	Prof. Anatomy, U. Mass. Med. Schl.	Loyd L. Glenn, Ph.D.	Guest Worker LNLC NINCDS	Aharony Lev Tov, Ph.D.	Visiting Fellow LNLC NINCDS	Richard F. Mayer, M.D.	Dept. Neurology, U. Maryland	James Toop, Ph.D.	Dept. Neurology, U. Maryland
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COOPERATING UNITS (if any) Dept. of Neurology, University of Maryland School of Medicine, Baltimore, MD Dept. of Anatomy, University of Massachusetts Medical School, Worcester, MA																
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SUMMARY OF WORK (200 words or less - underline keywords) This project is designed to provide information on the ranges and distributions of the electrophysiological and morphological characteristics of <u>alpha motoneurons</u> and of the interrelated mechanical, histochemical and morphological properties of the <u>muscle fibers</u> innervated by them (i.e., the muscle unit) in various hindlimb muscles in the cat. In some experiments, <u>motor unit</u> populations in normal animals are compared with those in animals after various conditioning treatments.																



## Project Description:

**Objective:** This project is designed to provide information about the populations of motor units that make up large limb muscle in mammals, including the electrophysiological and morphological characteristics of spinal cord motoneurons in relation to the mechanical, histochemical and anatomical properties of the muscle fibers (termed "muscle units") innervated by them. The neural and muscular elements are functionally inseparable, making the motor unit the quantum element in all motor behavior. The collection of detailed data about the characteristics of normal motor unit populations permit assessment of alterations that are produced by various experimental manipulations, including changes in mechanical demand or central nervous system lesions, or after axonal damage and reinnervation by foreign motoneurons.

**Methods Employed:** For the most part, analysis of motor unit properties is carried out in anesthetized cats using methods of intracellular recording and stimulation of individual spinal motoneurons to ensure functional isolation of individual motor units. Electrophysiological properties intrinsic to the motoneuron, and the quantitative and qualitative characteristics of synaptic inputs to the cell, are evaluated with conventional techniques. The mechanical properties of the muscle unit innervated by each cell is then assessed during stimulation of the motoneuron through the intracellular pipette electrode, with the innervated muscle attached to a force-measuring device under isometric conditions. Muscle fibers of individual motor units can be labeled by depleting intrafiber glycogen during prolonged stimulation of the motoneuron, permitting histochemical and morphological study of the muscle units belonging to physiologically-characterized individual units. Classification of motor units by type is done using methods and criteria described in earlier Annual Reports. Analysis of the anatomy of motoneurons innervating particular motor unit types has been done using intracellular injection of the tracer protein, horseradish peroxidase, which stains the entire intraspinal portion of individual cells. Animals are then perfused with dilute aldehyde fixative and the spinal cords subsequently cut into serial 50 - 75  $\mu$ m sections on a freezing microtome or a vibratome. HRP is visualized by reaction with diaminobenzidine enhanced by low concentrations of cobalt ions.

## Major Findings:

- A. Immunocytochemical Characteristics of Myosins in Muscle Units after Cross-reinnervation by Foreign Motoneurons.

This project has been reported in detail in the Annual Report for FY 1980. During FY 1981, frozen muscle tissue blocks from some of the experimental animals were examined by Dr. Geraldine Gauthier, of the Dept. of Anatomy, University of Massachusetts Medical School, using immunocytochemical methods to detect the presence of various subfragments of myosins associated with fast twitch or slow twitch muscle. Antibodies directed against the slow anterior latissimus dorsi (anti-ALD) of the chick react with type I (low myofibrillar ATPase) fibers in the normal cat soleus (SOL) and flexor digitorum longus (FDL). These type I fibers do not normally react with antibodies directed

against the N-terminal sequences of the alkali-1 (anti- $\Delta 1$ ) or alkali-2 (anti- $\Delta 2$ ) subfragments of fast twitch myosin. However, after the SOL muscle has been cross-reinnervated by the largely fast twitch population of  $\alpha$ -motoneurons in the FDL motor nucleus, the cross-reinnervated fibers exhibit reaction with the anti- $\Delta 2$  antibody but not with anti- $\Delta 1$ , and they continue to react with anti-ALD. Thus, the myosin in cross-reinnervated SOL muscles is only partially "transformed" by the foreign reinnervation. As reported earlier, the muscle units in cross-reinnervated SOL contract more rapidly than normal SOL units but they retain all other characteristics of type S muscle units, again signifying only partial transformation. In contrast, FDL muscle fibers cross-reinnervated by SOL motoneurons (all of which are normally type S) are completely transformed to type S by physiological criteria, and to type I fibers by ATPase histochemistry. The immunocytochemical results thusfar available indicate that such cross-reinnervated FDL fibers, which normally show a mosaic of histochemical and antibody reactivities, now virtually all react with anti-ALD but not with either anti- $\Delta 1$  or anti- $\Delta 2$ . Thus, the antibody studies re-enforce the physiological and conventional histochemical observation that cross-reinnervated FDL muscle is completely transformed by the foreign innervation but the cross-reinnervated SOL is not. The reason for the asymmetrical results of the two-nerve crossing paradigms is unknown. The data suggest that the SOL muscle of the cat is unusually resistant to trophic alteration by innervation or by drastic changes in its functional usage pattern, perhaps because of an intrinsic specialization not present among the fibers of heterogeneous muscles. Previous studies in this laboratory have also suggested that the muscle fibers in the cat SOL are in several respects unique, and significantly different from the slow twitch muscle units present in heterogeneous muscles. These immunocytochemical studies will continue in FY 1982, using additional material currently in freezer storage.

#### B. Effect of Spinal Cord Lesions on the Motor Unit Populations in the Medial Gastrocnemius of the Cat.

During FY 1981, data analysis was completed on histochemical material from a project begun in FY 1977 on the effect of complete spinal cord transections on the properties of the medial gastrocnemius (MG) muscle and its motor unit population. Detailed counts of histochemical muscle fiber types have shown that MG muscles in three cats with chronic spastic paraplegia, evaluated 6 months after total spinal section at the last thoracic segment, contain proportions of type I and IIA fibers, and of the corresponding type S and type FR motor unit types, that are considerably smaller than expected based on large survey samples from normal MG muscles. There was also a striking excess of fast twitch motor units with intermediate fatigue resistance (called type F(int)) and a similar, though not as marked, excess of the histochemical fiber type (called type IIAB) that appears to be associated with this unit type. The deviations in animals with complete spinal section were greater than the interanimal variations observed in normal cats. We conclude that chronic spastic paraplegia can produce a true alteration in the composition of the MG motor unit population, which was not found in previous studies of in conditions of prolonged muscle immobilization with atrophy, or in chronic compensatory hypertrophy. In order to arrive at this inference from the experimental observations, a number of

assumptions must be made, which involve the precision of the correlations between muscle fiber histochemistry and muscle unit type, the average innervation ratios among the different motor unit types and whether or not these can change with altered conditions, and the sampling probabilities within a diverse population of motor units. We have experimental evidence to constrain the first two issues but the problem of motor unit sampling has received little systematic attention. We have applied simple probabilistic models to motor unit sampling data available in LNLc in to assess how much of the observed variation between normal animals, and between such normal samples and samples from treated animals, can be explained simply because of random sampling errors. The hypergeometric distribution was used to develop probability tables for unit type frequencies in different sample sizes. Most of the data from normal animals fell within 95 percent confidence limits but the samples in all three of the spinal section cats were outside these limits in one or another respect, lending some further support to the conclusion that spinal section alters the MG motor unit population.

#### C. Anatomy of Motoneurons that Innervate Defined Types of Muscle Units.

We have accumulated a relatively large number of examples of motoneurons labeled intracellularly with horseradish peroxidase (HRP) and identified as innervating physiologically-defined types of muscle units as a result of a project on the anatomy of group Ia afferent fibers, described under project number Z01 NS 01686-13 LNLc. Over 50 cells of the triceps surae or plantaris muscles have been studied, including two  $\gamma$ -motoneurons. Axonal conduction velocity data is available for all cells, and afterhyperpolarization durations, input resistances and synaptic potential amplitudes have been measured in some. The analysis to date shows that the range in soma size (measured as the average diameter, taking largest and smallest somatic dimensions) fits exactly with data measured earlier in LNLc using retrograde transport of HRP to label  $\alpha$ - and  $\gamma$ -motoneurons. The average soma diameters of type FF and FR  $\alpha$ -motoneurons are similar (52.9  $\mu\text{m}$  and 53.1  $\mu\text{m}$ , respectively) and they have equivalent average numbers of main stem dendrites (11.9 and 12.6, respectively), but FF dendrites tend to be somewhat thicker than those of FR cells (average main stem diameters 7.8  $\mu\text{m}$  versus 6.7  $\mu\text{m}$ , respectively). Motoneurons of type S units have smaller average soma diameters (49.1  $\mu\text{m}$ ) and slightly fewer and thinner main stem dendrites (average 10.8 per cell with mean trunk diameter 6.3  $\mu\text{m}$ ). Thus, there is a general trend in motoneuron size such that the cells of FF units are, on average, the largest and the type S are the smallest, with type FR intermediate. There is, however, a large degree of overlap between the type-groups, so that motor unit type is not a very accurate predictor of motoneuron size. The two  $\gamma$ -motoneurons studied to date are much smaller than the  $\alpha$ -motoneurons and their somatic diameters (23  $\mu\text{m}$  and 29  $\mu\text{m}$ ) fit well with the second size peak found in retrograde HRP studies. It is of some interest that both  $\gamma$ -motoneurons possessed recurrent axon collaterals.

#### D. Histochemical Composition of Cat Leg Muscles.

The projects described under number Z01 NS 02080 (Neuron Activity During Locomotion) involve studies of the dynamic action (force production, length

changes and electromyographic activity) in several groups of cat hindlimb muscles. Most have not been studied in any detail for intramuscular anatomy and histochemical fiber composition and we continue to accumulate information about these aspects of neuromuscular design, concentrating on muscles and muscle groups that are under investigation by chronic implant techniques.

#### Significance to Biomedical Research and the Program of the Institute:

Analysis of the control of movement by the central nervous system requires consideration of the properties and functional specialization of motor units, since they are the quantal elements from which all skeletal movements are composed. Studies of the interrelation between the intrinsic properties of motor units (including both the motoneuron and muscle unit portions) and the organization of synaptic input to the same units have aided our understanding of the control problem and have suggested new avenues for research. In addition, elucidation of the detailed interrelation between the physiological, morphological and histochemical characteristics of muscle units in animal muscle has relevance to investigations of human neuromuscular disease, in which electromyography and muscle histochemistry play important diagnostic and research roles. There is growing evidence that the basic pattern of motor unit organization in animals and man is similar in principle. Studies of the effects on motor unit populations of altered usage, CNS lesions, and denervation - reinnervation in the cat have clear implications for the interpretation of clinical investigations and neuropathology in patients with neuromuscular disorders, peripheral neuropathies and CNS lesions.

#### Proposed Course of the Project:

Immunocytochemical analysis of the myosins in normal, self-reinnervated and cross-reinnervated FDL and SOL muscles will continue in FY 1982, in collaboration with Dr. Gauthier. Upon completion, selected portions of the remaining material may be subjected to gel electrophoresis to supplement the histochemical and immunocytochemical observations. The other aspects of this project have been completed. We will continue to accumulate HRP-labeled motoneuron material through FY 1982. Efforts are now being made to develop a practical approach to quantitative topological analysis of motoneuron anatomy, including estimates of dendritic domains, axes of symmetry and domains of intersection with afferent arborization, in addition to the more usual cell dimensions. Attempts thusfar have used only hand analyses and this will continue until such time as the detailed requirements for a computer-based analysis system are fully defined.

#### Publications:

Burke, R. E. Motor units in mammalian muscle. In: Sumner, A. J. (Ed.) The Physiology of Peripheral Nerve Disease. New York: W. B. Saunders 1980. pp 133-194.

Burke, R. E. Motor unit types: Functional specializations in motor control. Trends in the Neurosciences. 3:255-258, 1980.

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Mayer, R. F., Burke, R. E., Toop, J., Hodgson, J. A., Kanda, K. and Walmsley, B. The effect of long-term immobilization on the motor unit population of the cat medial gastrocnemius muscle. Neuroscience. 6:725-739, 1981.

Burke, R. E. Motor units in cat muscles: Anatomical considerations in relation to motor unit types. In: Rowland, L. P. (Ed.) Proceedings of MDA International Conference on Amyotrophic Lateral Sclerosis. New York: Raven Press. In press.







# ANNUAL REPORT

October 1, 1980 through September 30, 1981

## Laboratory of Neurophysiology

### National Institute of Neurological and Communicative Disorders and Stroke Table of Contents

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## ANNUAL REPORT

October 1, 1980 through September 30, 1981

Laboratory of Neurophysiology  
National Institute of Neurological and  
Communicative Disorders and Stroke

Henry G. Wagner, M.D.

Further effort was made to elucidate the mechanisms controlling the receptive fields of neurons. For this study, the retinal ganglion cell is particularly suitable because quantitative functional measurements can be easily made. Two methods of estimating the diameter of the field are commonly used. One is the sensitivity profile. The limit of the field being the limit of sensitivity. The second method determines the limit of area-intensity summation. The two methods often provide different values for the same cell. Our look into this problem disclosed that the receptive fields in the ganglion cells of carp belong to two classes of ganglion cells. One with a relatively small diameter and the other with a larger diameter. Each showed a "bell" shaped profile of sensitivity with highest sensitivity in center. However, this finding was not the explanation for the discrepancy. Our study also determined that under different stimulus conditions the area-intensity summation plot often provided different limits even in the same cell. Our interpretation now is that the receptive field diameter is a functional concept and can be different for different stimulus conditions. This may be the explanation for the paradox.

Earlier work on the cones of the salamander retina, found that annular illumination increased chloride conductance in the cone but that hyperpolarization suppressed this effect. This finding suggested a voltage-dependent sensitivity of the chloride channel. The present study was made to confirm this observation using a retina more favorable to this kind of study. The turtle retina was chosen since it permitted easier and more stable recording from cones. Annular illumination decreases the input resistance of turtle cones and results in a depolarizing response when the intracellular concentration of  $\text{Cl}^-$  is raised. Hyperpolarizing current blocks this synaptic response, while depolarizing pulses in darkness evoke potential and resistance changes similar to those associated with the surround effect. Furthermore, the response to annular illumination is transient and shows refractoriness. From these observations, it is concluded that the surround response of cones arises from the activation of a voltage and time-dependent  $\text{Cl}^-$  conductance.

Electrophysiological experiments using intracellular recording techniques and extracellular applications of neuroactive compounds have been performed on mouse spinal neurons and pituitary cells grown in tissue culture and on molluscan central neurons. The research has focussed on the factors that determine and regulate neuronal excitability. We have found in all of the molluscan and vertebrate neurons examined thus far that action potentials are generated only in axonal membranes but not in



somatic or dendritic membranes. We plan to examine whether or not this is a general property of neurons and produce superficially similar effects on cultured neurons.

We have begun to characterize several forms of chemical excitability revealed by pharmacological applications of these substances. We plan to examine the hypothesis that some pharmacologic actions of certain drugs are mediated through receptors for endogenous ligands.

Mammalian central neurons have been grown in tissue culture to study 1) immunohistochemically identified peptidergic and GABAergic neurons, 2) opioid peptide synthesis by some of these neurons, and 3) the biochemical characteristics of specific receptors for endogenous ligands. Sensory and spinal neurons stain specifically for nerve-specific enolase, while non-neuronal cultured cells do not. Some of the cultured neurons stain positively for either methionine or leucine-enkephalin, dynorphin, substance P, somatostatin or glutamic acid decarboxylase (GAD). Enkephalinergic neurons can synthesize and release methionine-enkephalin when incubated with labelled methionine. Binding studies have demonstrated the existence of several types of receptors on cultured neurons including those for GABA, opioid peptides and benzodiazepines. The results show the utility of using cultured nerve and muscle cells as model systems to study, with biochemical techniques, the synthesis of endogenous substances and the biochemical characteristics of their receptors.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 NS 02019-09 LNP
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less) Electrophysiology and Neuropharmacology of Simple Cellular Systems		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:   OTHER:	T.G. Smith J.L. Barker H. Lecar R. Canada K. Futamachi R. Study B. Dufy D. Mathers M. Jackson W. Vaughn W. Sheriff J. Mazzetta K. Saunders W. Bean	Section Chief Medical Officer Research Scientist Staff Fellow Staff Fellow Staff Fellow Guest Worker Visiting Fellow Post-doctoral Fellow Computer Specialist Computer Specialist Technician Technician Technician
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COOPERATING UNITS (if any) Laboratory of Biophysics, NINCDS; Research Services Branch, NIMH; R.N. McBurney, University of Newcastle School of Medicine, England; J. Bottenstein, Assistant Professor, UCLA; University of Bordeaux, School of Medicine		
LABORATORY Laboratory of Neurophysiology		
SECTION Section on Sensory Physiology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
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SUMMARY OF WORK (200 words or less - underline keywords) Electrophysiological experiments using intracellular recording techniques and extracellular pharmacologic applications have been performed on mouse spinal neurons and <u>pituitary cells</u> grown in tissue culture and on molluscan central neurons. The research has focussed on the mechanisms of how endogenous and exogenous ligands alter <u>neuronal excitability</u> . We have found that <u>endogenous substances</u> and <u>exogenous drugs</u> can produce superficially similar effects on cultured neurons. We have applied "fluctuation" and patch clamp analysis to one of the actions of transmitters and drugs and find that both natural and synthetic agents can open two-state Cl <sup>-</sup> ion channels. We have begun to characterize other forms of chemical excitability revealed by pharmacological applications of these substances. We plan to examine the hypothesis that some pharmacologic actions of certain drugs are mediated through receptors for endogenous ligands.		
3 LNP/IRP		

## Project Description

Objectives: Characterization of chemical and electrical forms of excitability on neuronal membranes and the modes of action of clinically important drugs in regulating excitability.

Methods Employed: Excitability is studied using intracellular recordings made from individual neurons with conventional, voltage clamp and patch clamp techniques. Endogenous substances and exogenous drugs are applied locally to the surface of individual cells using iontophoretic and pressure methods. Electrophysiological assays of membrane events induced by various substances can thus be made relatively easily with each cell serving as its own control. The accessibility of the neurons growing in monolayer culture and invertebrate ganglia permits long-term stable intracellular recordings which are critical for producing accurate measurements of membrane excitability. Experiments are designed to study membrane events with one of four levels of analysis: 1) single electrode membrane potential and conductance measurements; 2) double electrode voltage-clamp analysis of membrane current events 3) patch clamp measurement of current from small areas of membrane and 4) fluctuation or "noise" analysis of membrane current fluctuations. The analysis is performed off-line with the aid of a digital computer.

Light microscopic studies employ conventional and novel light microscopes. Fluorescently labeled probes are employed to locate receptor and binding sites and ionic channels.

Major Findings: 1. Pharmacology of natural substances and clinically important drugs in cultured central neurons. Virtually all spinal cord cells grown in culture respond to each of the neutral amino acids glycine,  $\beta$ -alanine and  $\gamma$ -aminobutyric acid (GABA), but each responsive neuron shows a non-uniform distribution of responses to the amino acids. These results suggest the development of "hot spots" or clusters of receptors on the cell surface. Responses elicited by application of amino acids to neuronal processes are frequently complex, while those evoked at the level of the cell body are always inhibitory to cell excitability. The inhibitory response consists of an increase in membrane conductance to  $\text{Cl}^-$  ions. We have studied the elementary events underlying the increase in  $\text{Cl}^-$  ion conductance using the technique of fluctuation analysis. We have used fluctuation analysis to estimate the electrical dimensions of elementary events associated with membrane current responses to the neutral amino acids.

Glycine-activated channels are approximately twice the conductance and one-quarter the lifetime of those activated by GABA, while channels activated by  $\beta$ -alanine are significantly different from those opened by either glycine or GABA. Thus, the charge transfer occurring during an elementary  $\text{Cl}^-$  ion channel event activated by the different amino acids is unique for each amino acid. The energy required for a channel to close is the same for GABA and glycine channels. The unique properties of the  $\text{Cl}^-$  ion channels activated by the three naturally occurring

amino acids implies that physiologically elaborated synaptic events mediated by the amino acids have unique properties if other factors (e.g., metabolic) are unimportant relative to channel properties in shaping the synaptic signal.

In collaboration with the Laboratory of Biophysics, IRP, NINCDS, we have examined the electrical properties of elementary ion channel events activated by GABA and the GABA analogue muscimol. The results show that both substances cause inward current jumps under recording conditions where the macroscopic current response is also inward. The current jumps are step-wise and rectangular. They are considered to reflect the activity of individual ion channels activated by the agonists. The amplitudes of the current jumps appear to have a unimodal distribution, suggesting they come from one population of ion channels. The channel conductance calculated from direct patch recording agrees closely with estimates made from noise analysis. Time-duration histograms of the current jump durations, from which the average duration of an elementary event can be calculated, are often better-fitted by two exponentials. These results indicate that at least in some microscopic membrane patches and at low agonist concentrations, the kinetics of GABA and muscimol-activated channels are complex. We have applied fluctuation analysis to GABA and muscimol responses obtained under the same low agonist concentrations and recording conditions used to make the recordings from microscopic patches. We have now found evidence that at low agonist concentrations the kinetics of channel activities are indeed more complex than had been observed at higher concentrations. The kinetics of channel activity estimated from spectral analysis of responses evoked by low concentrations of agonist agree well with the results obtained in patches. We plan to try to resolve the complexity of the kinetics.

We have also applied fluctuation analysis to structural analogues of GABA to begin to understand how structure determines function. The results show that the average conductance of a channel activated by an analogue is not significantly different from that activated by GABA while the durations of analogue-activated channels are all significantly different from that of GABA. Although we have not completed a detailed study of comparative potencies of the different analogues, preliminary results show good agreement between the effectiveness of an analogue in evoking macroscopic current responses and the duration of the microscopic conductance event.

We have correlated the data on channel duration with previously published values on the potency of the same GABA analogues in displacing labelled GABA from receptor sites on frozen rat and human brain membranes. The correlation coefficients are all equal to, or greater than 0.90 with binding data from six independent studies. All of these correlations are significant at the .01 level. The results suggest that the biochemical and electrophysiological assays measure some parameter(s) common to GABA receptor function. It is thus possible that the analogues are agonists at GABA receptor sites similar to those used in "Na<sup>+</sup>-independent" binding studies. The present results do not yet allow us to specify exactly how the binding of the agonist structure to the GABA

receptor determines the duration of the conductance change, since neither the biophysical nor binding experiments distinguish between binding and conformational equilibria.

Less than 10 percent of cultured cells derived from dorsal root ganglia respond to GABA with a  $\text{Cl}^-$ -dependent depolarization. These cells do not respond to either glycine,  $\beta$ -alanine (or glutamate). Fluctuation analysis of the GABA responses reveals single channel estimates similar to those recorded on spinal cord cells.

We have also found that each of the neutral amino acids can antagonize the membrane response to another amino acid on spinal cord cells. On a number of spinal cord cells we have found a high and significant correlation between response amplitudes to GABA and glycine evoked on different parts of the same cell. The results suggest that the two amino acid receptors may be coupled to the same  $\text{Cl}^-$  ion channel. Future experiments will be directed at examining the organization of receptors and channels on central neuronal membranes.

Several classes of clinically important drugs have been applied to cultured spinal neurons. Bath application of agents which induce convulsions in vivo, including picrotoxin, pentylenetetrazole, penicillin, bicuculline, and strychnine cause paroxysmal depolarizing events in tissue cultured neurons. These events involve intermittent 20-30mV depolarizations which elicit high frequency repetitive spike firing. Under voltage clamp the depolarizations appear to consist of many discrete inward-going synaptic-like current events which invert in polarity between -20 and 0mV. The depolarizing events are dependent on extracellular  $\text{Ca}^{++}$  and blocked by tetrodotoxin. We are presently doing fluctuation analysis of the synaptic-like events to see if they can be described by the same equation which accounts for the kinetics of two-state ion-channels, and whether the events evoked by different convulsants have similar properties at this level of membrane assay.

We have further found that three classes of drugs which are anticonvulsant in vivo, including benzodiazepines, barbiturates and hydantoins are able to attenuate the amplitude and duration of the paroxysmal events in a reversible manner. The anticonvulsant activities are associated either with an elevation in the threshold for spike generation or a depression in the ability of a cell to fire repetitively. Local application of convulsants and anticonvulsants to individual cells reveals that five different types of convulsants including picrotoxin, bicuculline, penicillin, pentylenetetrazole, and strychnine all lower the threshold for single and repetitive spike firing while the three classes of anticonvulsant elevated threshold for single and repetitive spike firing. Preliminary results show that the anticonvulsants can reverse the effects of the convulsants on electrical excitability on the same cell. These results suggest that regulation of electrical excitability is an important site of drug action. Presumably the changes in threshold would contribute to the clinical effects of the drugs.



We have also found that lowering of threshold for single and repetitive spike firing can occur following local application of nanomolar concentrations of GABA on about 20 percent of the cells studied. We have found a good correlation among those cells responding to bicuculline and those responding to GABA suggesting that bicuculline may actually be a GABA agonist at this site. Our next step will be to complete some of the phenomenological details before proceeding to study the underlying mechanisms.

A second site of action of clinically important drugs is associated with modulation of amino acid-mediated  $\text{Cl}^-$  ion conductance. The convulsants depress conductance responses evoked by GABA and glycine. Fluctuation analysis of these interactions shows that while picrotoxin, bicuculline and strychnine depress amino acid responses, they do not change the electrical properties of single channels activated by amino acids. One interpretation of these results is that these convulsants depress the frequency of successful ion channel events so that fewer events contribute to the amino acid-induced current response, but those that do contribute have electrical properties similar to those observed under control conditions. Pentylenetetrazole and penicillin appear to shorten the average duration of GABA-activated channels without altering their average conductance. It is not yet clear whether the changes in estimated channel properties can account for the depression of GABA responses by these convulsants.

Further study of the effects of the anticonvulsant phenobarbital reveal that the drug does not potentiate GABA responses at clinically relevant concentrations (20-100  $\mu\text{M}$ ). We have examined the molecular mechanisms underlying the potentiation of GABA responses by the anesthetic (-)pentobarbital and the anticonvulsant diazepam. The results show that both drugs potentiate GABA responses by altering the kinetics, and not the conductance of  $\text{Cl}^-$  ion channels activated by GABA. The barbiturate prolongs the average duration of an individual channel and decreases the probability of a channel event occurring. The benzodiazepine markedly increases the number of channel events activated by GABA with little change or a modest increase in the average duration of an individual channel event. Both types of drug action would effectively increase the change transfer occurring during a  $\text{Cl}^-$  ion channel event activated by GABA. Thus, using fluctuation analysis we have been able to account in a quantitative manner for the potentiating effects of these two classes of clinically important drug. Exactly how these drugs alter the kinetics of GABA-activated  $\text{Cl}^-$  ion channels needs to be examined. Pentobarbital increases the affinity of GABA for receptor sites and retards the unbinding of GABA from receptor sites. These activities may be relevant to the results described above from electrophysiological assays of GABA receptor functions.

We have also found that the stereoisomers of the anesthetic pentobarbital and the benzodiazepines diazepam and flurazepam can produce transmitter-like effects on cultured spinal neurons. The (+) isomer of pentobarbital produces a predominantly excitatory action over the low-to-moderate range of concentrations. At higher concentrations the

isomer indirectly inhibits excitability by increasing membrane conductance, presumably to  $\text{Cl}^-$  ions. The (-) isomer of pentobarbital is very weakly excitatory at low concentrations and strongly inhibitory at moderate to high concentrations, inhibiting excitability by increasing membrane conductance to  $\text{Cl}^-$  ions. Flurazepam and diazepam also activate  $\text{Cl}^-$  ion conductance in some cells. Under voltage clamp both barbiturate and benzodiazepine drugs induce a membrane current response associated with visible fluctuations in the current trace. Spectral analysis of these fluctuations shows that they are well fit by a single Lorentzian equation. The estimated average duration of these barbiturate-induced channel events is about five times that estimated for GABA-activated channels on the same cell, while those activated by diazepam have the same duration. The conductance of both of these channels is similar to that estimated for GABA. Close examination of the baseline spectra reveals that they are best fit by a  $1/f$  relationship with no detectable evidence of an incipient Lorentzian term indicative of the presence of ambient GABA. Thus, despite the fact that the drugs each activate a  $\text{Cl}^-$  ion conductance like GABA, it is not clear how they are "GABA-mimetic". The drugs do not appear to be acting by potentiating ambient GABA. One interpretation is that they can engage GABA receptors and simultaneously modulate the receptor-activated  $\text{Cl}^-$  conductance. Alternatively, they may engage receptors for an endogenous ligand other than GABA, which are coupled to a  $\text{Cl}^-$  conductance mechanism or they might be able to activate some  $\text{Cl}^-$  channel mechanisms directly.

2. Analysis of spontaneously occurring membrane events in cultured spinal cord and sensory neurons. Fluctuation analysis of the majority of spinal cord cells recorded in the presence of tetrodotoxin and elevated magnesium ions (to block synaptic activity) shows that resting membrane current fluctuations can be described by a relatively simple " $1/f$ " spectrum. Such a spectrum has been observed in other excitable membranes and is thought to reflect the summed activities of ionic conductance mechanisms associated with the resting membrane. These simple spectra are present over the  $-40$  to  $-100$  mV range of membrane potential. Voltage steps to potentials more depolarized than  $-40$  mV evoke outward current responses associated with an increase in membrane conductance and membrane current variance. Spectral analysis of membrane current fluctuations occurring at these depolarized potentials reveal spectra which depart from " $1/f$ " behavior. These have yet to be fully analyzed. They may represent the activities of  $\text{K}^+$  channels activated at levels of membrane potential more depolarized than resting membrane potential.

A minority of cells possess either discrete synaptic-like current events and/or fluctuations in baseline membrane current. Fluctuation analysis of the baseline activity reveals spectra characteristic of those observed with chemical excitability. The discrete baseline events may reflect quantal synaptic currents, while the other activities associated with baseline recordings may represent a form of tonic transmitter release. The spectra most commonly associated with naturally occurring events resemble those observed during membrane current fluctuations induced by glycine. In several cells we have recorded spontaneously occurring synaptic currents whose time constant of decay is close to that

estimated for channels activated by GABA in the same membrane. We plan to continue this part of the study by comparing synaptic signals in cultured neurons with estimates of the elementary properties of amino acid and peptide-activated channels in order to try to identify the substances mediating these signals.

We have also studied the baseline activities of cultured sensory neurons derived from the dorsal root (sensory) ganglion. About 50 percent of these cells have electrically quiet membranes devoid of any discrete events or membrane current fluctuations. On the remaining half we have observed both discrete voltage events and fluctuations in the resting membrane potential. These spontaneously occurring activities are always hyperpolarizing in direction and are insensitive to tetrodotoxin. They disappear abruptly as the membrane potential is hyperpolarized and cannot be reversed in polarity. Superficially the hyperpolarizations resemble synaptic potentials and are dependent on the presence of  $\text{Ca}^{++}$  ions. Under voltage clamp the potentials are replaced by outward-going, discrete current events and fluctuations, in membrane current. These current activities disappear as the membrane potential is hyperpolarized to about  $-70\text{mV}$  and cannot be reversed in polarity.

Amplitude and interval histograms of the voltage and current activities reveal the presence of elementary-sized events which are not precisely distributed in either a Gaussian or Poisson manner. The apparent amplitude of the elementary current event is  $40\text{pA}$  at  $-45\text{mV}$ , giving an estimated elementary conductance of about  $1.3\text{nS}$ . This is about 500-fold greater than amino acid-activated  $\text{Cl}^-$  ion channel events. All of the discrete events and fluctuations are blocked in a reversible manner by local application of tetraethylammonium ions, which block  $\text{K}^+$  conductance in a variety of excitable membranes. The events are also sensitive to extracellular applications of  $\text{Co}^{++}$  and D-600, both of which antagonize  $\text{Ca}^{++}$  conductances in other membranes. Spectral analysis of these events at the resting potential shows that the fluctuations are well fit by a single Lorentzian equation similar to that used to describe chemical excitability at synapses. From the cut-off frequency of the Lorentzian equation we estimate the average duration of an event to be about  $10\text{msec}$ . At more depolarized potentials the power in the spectrum declines as more than the square of the frequency.

We interpret the hyperpolarizing events as reflecting activation membrane conductance to  $\text{K}^+$  ions. The conductance also appears to be dependent on extracellular  $\text{Ca}^{++}$  ions. The ionic conductance is only activated over a narrow range of membrane potential ( $-60\text{mV}$  to  $-20\text{mV}$ ). Since there is little tendency for the events to diminish in frequency at the resting membrane potential, it is likely that this conductance contributes to the resting membrane properties. In fact, those sensory cells exhibiting this phenomenon have resting potentials significantly greater than cells without the events. Any tendency to excite cells possessing this  $\text{K}^+$  conductance will further activate this conductance, thus hyperpolarizing the cell. Naturally occurring substances which

depress this conductance would depolarize the cell and lead to excitation. There is as yet no evidence that this conductance is sensitive to amino acids or acetylcholine. Morphological studies at the light and electron-microscopic levels should provide some necessary evidence to distinguish between either an endogenous or exogenous source. Since the events occur in cultures devoid of spinal cord cells, it is unlikely that these cells are the source. Our goal is to understand the role of this conductance in the physiology of sensory cells.

### 3. Electrophysiology of GH3/6 cells

We have begun to study naturally occurring membrane events on GH3/6 cells, a clone line of pituitary cells which secrete prolactin. The majority of cells studied thus far are electrically excitable. A small percentage of these cells spontaneously generate action potentials and also possess spontaneous fluctuations of the baseline membrane potential. Analysis of the membrane properties of these cells shows that virtually all of the cells possess non-linear, steady-state current-voltage relations. As membrane potential is stepped from  $-60\text{mV}$  to more depolarized levels there is a rapid outward current response associated with an increase in membrane conductance. The initial changes evoked by such steps decay within seconds, leaving a steady-state outward current conductance. This steady-state membrane current is associated with a corresponding increase in membrane current variance. In conventional (unclamped) recording conditions, tetraethylammonium ions (TEA) depolarize GH3/6 cells, inducing spontaneous action potentials. TEA also linearizes the steady-state current-voltage curve. The results suggest that a voltage-dependent  $\text{K}^+$  conductance determines the resting membrane potential of the cell much like the aforementioned  $\text{K}^+$  conductance in sensory neurons.

Fluctuation analysis of the membrane current at different membrane potentials shows that there is a potential-dependent increase in membrane current variance. The variance is fit by a  $1/f$  spectrum at resting and more hyperpolarized levels and a more complex spectrum ( $1/f$  and  $1/f^2$ ) at more depolarized levels. TEA completely blocks the increase in variance, as does  $\text{Co}^{++}$  and D600. Presumably, the spectra reflect the complex kinetics of  $\text{K}^+$  ion channels, which are activated over a prescribed range of membrane potentials and involve a  $\text{Ca}^{++}$  requiring step.

Nanomolar amounts of the tripeptide thyrotropin releasing factor cause the release of prolactin from pituitary cells *in vivo* and from the GH3/6 clone cells. We have begun to examine the effects of nanomolar concentrations of TRH on the electrical excitability of GH3/6 cells. TRH induces a short-lasting hyperpolarization of the resting membrane potential and a longlasting increase in fluctuations of membrane potential. Associated with the increase in membrane current variance is a steady outward current. The non-linear steady-state current-voltage curve observed in control shows a time- and voltage dependent change in its non-linear properties. These complex changes induced by the peptide hormone are under careful analysis.



4. Molluscan Neuron Physiology and Pharmacology. By employing the newly developed patch-clamp technique, small discrete areas of neuronal membrane of individual cells can be studied in isolation from the rest of the neuronal surface. Upon investigating the giant neurons of the sea hare, *Aplysia californica*, we have found that, while an action potential can be recorded from electrodes placed in the soma of these cells and the somatic membrane possesses an inward sodium current under voltage clamp, the somatic membrane of these cells does not generate an action potential under physiological conditions. Rather, the action potential is actively generated in the axonal membrane of these neurons and the somatic membrane acts as a passive return pathway for the flow of current during an action potential. The reason that the soma is passive during a spike, while having sodium channels, is that the density of the channels in the membrane is too low - only about .1 of the channel density in the axon. This is consistent with one finding that at least 0.2-0.3 of the sodium channels in the axon are required to support an action potential.

These experiments raise the question as to the generalizability of active axons and passive somata in all neurons, a question of considerable importance in the control of neuronal excitability. Preliminary studies on cultured spinal neurons indicate that the dendrites and probably the somas of these neurons do not generate action potentials but spikes occur only in the axons and possibly the axon hillocks.

There has been a long-standing controversy over the ionic conductances involved in a form of oscillatory neuronal activity known as bursting pacemaker potential activity. The argument is whether one of these conductances is a sodium or a calcium conductance. One datum against the sodium hypothesis is that the spontaneous oscillatory activity and the voltage clamp inward current are not blocked by concentrations of a sodium channel blocker, tetrodotoxin, (TTX) which blocks the sodium action potential. We have found that a 2-3 fold increase in TTX concentration does abolish the oscillations and the inward current reversibly, thus showing our sodium hypothesis to be correct.

A newly identified group of neurons in molluscan ganglia, which normally display only randomly firing infrequent spikes, can be made epileptogenic by several procedures. These include replacement of extracellular calcium and strontium and the addition of certain drugs (e.g., picrotoxin). The epileptogenic activity is characterized by randomly occurring, large depolarizing shifts in membrane potential and a rapid burst of spikes. Under voltage clamp, the untreated neuron shows an all-positive current-voltage curve. On the other hand, the epileptogenic neuron has a region of negative slope and is N-shaped. In addition, antiepileptic drugs (e.g., the hydantoins) return to normal electrical activity and remove the region of negative slope. Analysis of these changes shows that they are the membrane basis for the neuron becoming epileptogenic and the mode of actions of the antiepileptic drugs. Thus while synaptic activity and loci play an important role in the development of seizures and in the action of antiepileptic drugs (see below),



these experiments suggest that non-synaptic membrane mechanisms may play an important and fundamental role in epileptogenesis.

5. Fluorescence Studies. Using a new fluorescent microscope we have constructed, we have recently begun to demonstrate the localization of calcium binding sites in tissue cultured spinal neurons by employing the phenomenon of resonant energy transfer (RET). The rare earth element terbium is naturally fluorescent when stimulated with long wavelength UV light. Terbium can also displace calcium from its binding sites. When those binding sites are proteins, and the proteins are stimulative with short UV light, the energy absorbed by the proteins is transferred to the terbium by RET and re-emits the energy by fluorescence at terbium's characteristic wavelength. Thus by treating the neurons with terbium, stimulating them with the appropriate light and visualizing the terbium fluorescence, the calcium binding sites can be located. That the structures seen are, in fact, calcium binding sites is demonstrated by displacing the terbium and losing fluorescence with high concentrations of calcium.

We plan to continue this research by enumerating the various calcium binding sites and investigating what factors (other ions, pH, membrane potential, etc.) affect or regulate them.

6. Defined tissue culture media. A major problem with tissue cultured CNS neurons is the variability of the preparation. One of the important determinants of this variability probably derives from the use of horse serum in the growth media. By using a variety of additives (hormones, minerals, etc.) that have previously been shown to support neuroblastoma cells successfully in culture, we have grown tissue cultured neurons in serum-free media for periods up to two months. While the cultures still have to be started in a media with serum, they can be weaned from serum within days. Anatomically and electrophysiologically these cultures mature as well or better than cells grown in serum.

Significance to Biomedical Research and the Program of the Institute: Identifiable molluscan neurons and dissociated cultures of the mammalian central nervous system are proving to be an extremely useful preparation to study the physiology and pharmacology of central neurons.

We have focused our efforts, in part, on the localization and the factors which regulate electrical excitability in neurons. Our results suggest that such excitability is restricted primarily to axons and that somata are passive under physiological conditions. If this situation is generalizable to all neurons, it has important implications for our understanding of neuronal function. For example, if the situation were otherwise, i.e., if somata and dendrites had active spike membranes, the influence of any synaptic activity arriving before or during a spike would be completely shunted out by the large spike conductance and have no further affect on membrane potential activity. But by restricting spike

activity to areas that do not receive synaptic input - the axons - there can be a continuous, ongoing modulation of membrane potential activity and of neuronal output.

We have also been focussing on several aspects of receptor pharmacology, examining the membrane effects of amino acids, peptides, purines, pyrimidines, benzodiazepines and barbiturates. We have been able to resolve details of the cellular pharmacology of these effects beyond that which has been shown in vivo. Our research has focused on receptor and membrane pharmacology since this aspect of cell-to-cell communication is relatively easily studied and yet plays a crucial role in intercellular communication and cell excitability. It is clear from these initial studies that a variety of clinically important drugs affect receptor-coupled and membrane changes in excitability and that these changes may well be one of the bases for their pharmacologic effects in the CNS. The results may thus help to provide a more solid scientific basis for the actions of these clinically important drugs.

Our recently begun experiments employing optical techniques promises to provide us with new methods of investigating the function of normal and diseased neurons. Particularly exciting is the prospect of gaining new insights into two ubiquitously important physiological ions - calcium and sodium

Proposed course: The project will continue to examine the physiology of individual neurons and of intercellular communication in the nervous system and the pharmacology of individual neurons and of central receptors and membranes.

The physiological experiments will continue to exploit the patch clamp technique in investigating both electrical and chemical excitability as well as interneuronal communication in nerve cells. In addition, we plan to establish a collaboration with Dr. S. Arch of Reed College, who has purified several neurohormones known to play a role in egg-laying activity in Aplysia. We will use these hormones to identify their target neurons and to elucidate their mechanisms of action at the membrane level. Neurohormones are being recognized as an increasingly important mechanism of regulating neuronal activity.

The neuropharmacology will proceed at the three aforementioned levels of analysis. Structure - activity - relationships of benzodiazepine and barbiturate actions on central neurons, single channel level analysis of receptor function, and correlation of synaptic events with estimates of elementary events are three particular areas of future focus. Such experiments should provide more meaningful and quantitative analyses and a better understanding of the membrane mechanisms underlying the actions of neuroactive substances. Much of the research carried out thus far has utilized unidentified spinal cord and dorsal root ganglion cells. An important advance that can be made will involve experiments with identified cell types in culture. Three lines of investigation undertaken for

this purpose are 1) the enrichment of cultures for specific cell types and 2) the application of immunohistochemical techniques for identifying vital neurons without the use of fixation techniques and 3) the development of defined media for growing tissue-cultured cells.

The fluorescent studies will proceed to identify those neuronal structures which have calcium binding sites, to locate the sites of sodium spike conductances and to investigate the factors that control and regulate them.

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COOPERATING UNITS (if any) LCS, NIMH: LBC, NIADDK																																						
LAB/BRANCH Laboratory of Neurophysiology																																						
SECTION Section on Sensory Physiology																																						
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																																						
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1	OTHER: 0.5																																				
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																																						
SUMMARY OF WORK (200 words or less - underline keywords) <p>           Mammalian central neurons have been grown in tissue culture to study 1) immunohistochemically identified <u>peptidergic</u> and <u>GABAergic</u> neurons, 2) <u>opioid peptide synthesis</u> by some of these neurons, and 3) the biochemical characteristics of specific receptors endogenous ligands. Sensory and spinal neurons stain specifically for <u>nerve-specific enolase</u>, while non-neuronal cultured cells do not. Some of the cultured neurons stain positively for either <u>methionine</u> or <u>leucine-enkephalin</u>, <u>dynorphin</u> substance P, <u>somatostatin</u> or <u>glutamic acid decarboxylase</u> (GAD). Enkephalinergic neurons can synthesize and release methionine-enkephalin when incubated with labelled methionine. Binding studies have demonstrated the existence of several types of receptor on cultured neurons including those for GABA, opioid peptides and benzodiazepines. The results show the utility of using cultured nerve and muscle cells as model systems to study, with neurochemical techniques, the synthesis of endogenous substances and the biochemical characteristics of their receptors.         </p>																																						

16 LNP/IRP



Objectives: The objective of this research is to gain insight into the (1) development, dispositions and functionality of central neuronal membrane receptors, (2) mechanisms of peptide synthesis, and (3) physiology of neurons identified immunohistochemically.

Methods Employed: The presence of receptors stereospecific for particular agonists is investigated using conventional receptor binding assay techniques. The mechanisms of peptide synthesis are studied by incubating cultures with radioactive precursor amino acid, extracting radioactively labelled peptides and submitting these to a multi-step purification procedure. Immunohistochemical identification of neurons containing specific antigens is carried out using conventional immunohistochemical fluorescence and "PAP" techniques applied to neurons grown on coverslips in culture.

Major Findings: 1. Immunohistochemistry of cultured neurons. A small fraction of the cells which grow in culture stain positively for nerve-specific enolase, an enzyme marker specific for nerve cells. Most of the cells in culture (fibroblasts and other background elements) do not stain for the enzyme. Intracellular recordings from those cells which stain positively for the enzyme showed that these cells possessed membrane properties characteristic of nerve cells in vivo, including excitability and spontaneous synaptic activity. Some elements whose morphology resembles the enolase-positive neurons can be stained, using immunohistochemical methods, for either of five peptides (substance P, somatostatin, dynorphin, leucine or methionine enkephalin) or glutamic acid decarboxylase, the enzyme important in the synthesis of GABA. These elements are presumed to be nerve cells. They do not appear to have distinctive morphologies. We are currently comparing the anatomical disposition of GAD-positive elements on cultured spinal neurons with the functional effects of GABA on the membrane properties of the cell invested with GAD-positive structures. Do GAD-positive structures invest nerve cells in a specific way and how is this investment related to GABA effects on membrane excitability?

2. Peptide synthesis. Spinal cord and brain cultures incubated in radioactive methionine synthesize and secrete methionine-enkephalin. The baseline observations should allow us to ask questions regarding regulation of synthesis and release. One of the next projects will be an examination of precursor-product relationships in cultured neurons. What is the time-course of synthesis? What regulates synthetic rate? Another project will focus on the secretory mechanisms associated with peptide and non-peptide release. What peptides and peptide fragments are released? Are they all functional and if so how do the functions differ?

3. Receptors on cultured cells. Binding assays using labelled ligands including benzodiazepines, GABA and opiates show the presence of stereospecific, saturable binding sites for each type of substance. The functional nature of the binding sites for the drugs has begun to be

approached with electrophysiological measurements of membrane events associated with the pharmacological actions of the drugs on individual nerve cells. What is the developmental biology of the three types of opioid peptide receptor ( $\mu$ ,  $\kappa$ ,  $\delta$ ) and how are these receptors related to the three types of chemical excitability?

Significance to Biomedical Research and the Program of the Institute: Cultured mammalian neurons appear to be a useful system to study receptor pharmacology and peptide synthesis with biochemical techniques. The prime advantages of the preparation are 1) the lack of diffusional barriers to radioactive ligands and precursors and 2) the opportunity to carefully control the extracellular environment. Demonstration of peptide synthesis *in vivo* has been all but impossible owing to the presence of physical barriers and uptake systems. Likewise binding properties of receptors from *in vivo* material utilize fractionated membrane suspensions, while the binding experiments in culture can use an intact monolayer of cells. The results achieved thus far contain important baseline observations upon which future questions will be predicated. What regulates the appearance of particular receptors? Are they inserted as completed units or do they mature into functional units while in the membrane? Where are they distributed on nerve cells? How are peptides synthesized and what regulates their synthesis? Finally, the combination of immunohistochemistry with electrophysiology should allow us to examine the physiology of central peptidergic and GABAergic neurons.

Even incomplete answers to the questions raised above will advance our knowledge of the developmental biology of receptors, peptide synthesis and the physiology of identified neurons, since little hard data exists in any of these areas today.

Proposed Course of the Project: The three projects briefly outlined - developmental biology of specific receptors, peptide synthesis and amino acid and peptide immunohistochemistry - will proceed as deliberately as possible. Once baseline observations have been obtained, the first generation of appropriate and important questions will be asked. Some of these questions have been stated in various sections of this report.

#### PUBLICATIONS

D.E. Schmechel, M.W. Brightman and J.L. Barker: Localization of neuron-specific enolase in mouse spinal neuron grown in tissue culture. Brain Research 181: (1980) 391-400.

J.F. McKelvy, C-J Kin, L. Chang, P. Joseph-Bravo, J-L. Charli, M. Pacheco, M. Paulo, J. Neale and J.L. Barker: Biosynthesis of Brain Peptides in Brain Peptides: A New Endocrinology, A.M. Grotto, E.J. Peck, and A.E. Boyd, eds., Elsevier/North Holland, pp. 182-196 (1979).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02331-04 LNP						
PERIOD COVERED October 1, 1980 to September 30, 1981								
TITLE OF PROJECT (80 characters or less)  A Study of the Complex Receptive Field Properties of Turtle Retinal Neurons								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%;"> <tr> <td style="width: 33%;">PI: Henry G. Wagner</td> <td style="width: 33%;">Chief</td> <td style="width: 33%;">LNP NINCDS</td> </tr> <tr> <td>Prof. P.L. Marchiafava</td> <td>Senior Investigator</td> <td>LNP - CNR</td> </tr> </table>			PI: Henry G. Wagner	Chief	LNP NINCDS	Prof. P.L. Marchiafava	Senior Investigator	LNP - CNR
PI: Henry G. Wagner	Chief	LNP NINCDS						
Prof. P.L. Marchiafava	Senior Investigator	LNP - CNR						
COOPERATING UNITS (if any) Laboratory of Neurophysiology CNR Pisa, Italy								
LAB/BRANCH Laboratory of Neurophysiology								
SECTION Section on Neuronal Interactions								
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205								
TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.2	OTHER: .1						
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS								
SUMMARY OF WORK (200 words or less - underline keywords) In the <u>turtle retina</u> , only about one third of the <u>intracellularly</u> recorded <u>ganglion cells</u> show color dependent responses. These cells receive only <u>bipolar</u> input and have been designated Type A. Their dendritic trees are monolayered in the <u>innerplexiform layer</u> . It is believed that their mechanism for color dependency is primarily based on interactions between their <u>cones</u> and <u>horizontal cells</u> . The remaining intracellularly recorded ganglion cells are not influenced by color and show an additional input from the <u>amacrine</u> cells. They have been designated Type B. Their dendritic trees are diffusely spread throughout the innerplexiform layer.								

## Project Description:

Objectives: To characterize the receptive field properties of certain neuron cell types in the turtle retina.

Methods Employed: Intracellular and extracellular recordings with glass microelectrodes of the electrical potentials produced by the retinal neurons in response to light stimuli are employed. Intracellular injection of dyes is also accomplished through the same electrodes so that following histological processing the structure of these cells may be studied by light and electron microscopy and correlated with the function determined electrophysiologically.

Major Findings: A collaborative study of the spectral sensitivities of amacrine, bipolar and ganglion cells of turtle was made. Using intracellular microelectrodes, cells were injected with horseradishperoxidase after recording, processed, mounted and examined microscopically for study of the structure of the neuron injected. Dendritic trees of these cells were photographed and traced with respect to the innerplexiform layers (IPL) to determine level of synaptic contacts. Two classes of ganglion cells could be distinguished. Type A shows color opponency. Its spectral max are in the red and in the green; its dendrites are monolayered in the IPL. Type B does not show color opponency with spectral max only in the red. Its dendrites are diffusely layered in the IPL.

Retinal ganglion cells in the streak of the turtle retina show orientation specificity; slits of light when shone upon the retina at the preferred orientation elicit a vigorous response but when shone orthogonal to this orientation evoke little response. Horizontal cells show a similar effect but to a lesser degree. Anatomical studies in the turtle retina using the Golgi technique show that some horizontal cells and some ganglion cells have non-uniform dendritic trees which are up to 2 times longer than they are wide and are oriented parallel to the streak.

Significance to Biomedical Research and the Program of the Institute: The neurons of the retina have been observed to have quite complex receptive fields. Even though these cells have been studied for over two decades, the mechanisms by which these receptive fields are generated have yet to be elucidated. Our finding of cell properties in the turtle retina may allow the determination of these mechanisms because all types of retinal neurons can be impaled with intracellular electrodes. The use of intracellular staining will allow the determination of structural-functional correlations in these mechanisms.

Proposed Course of the Project: This project will be terminated with the publication of a report cited below.

## PUBLICATIONS

P.L. Marchiafava and H.G. Wagner: Interactions leading to color opponency in ganglion cells of the turtle retina. Proc. Roy Soc. B 211: 261-267, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02293-05 LNP
PERIOD COVERED July 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Neural Integration and Processing in the Mammalian Visual System		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <div style="display: flex; justify-content: space-between;"> <span>PI: H.G. Wagner</span> <span>Chief</span> <span>LNP NINCDS</span> </div>		
COOPERATING UNITS (if any) M.L. Wolbarsht, Duke University J. Ringo, Duke University		
LAB/BRANCH Laboratory of Neurophysiology		
SECTION Section on Neuronal Interactions		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <div style="text-align: center;">0.1</div>	PROFESSIONAL: <div style="text-align: center;">0.1</div>	OTHER: <div style="text-align: center;">0</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS   <input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS         </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords) A study of the <u>receptive fields</u> of <u>ganglion cells</u> in the <u>cat</u> has been made. Using strong <u>chromatic adaptation</u> , the extent and character of the receptive fields of the several spectral components converging on the ganglion cells has been examined. Central responding areas appear to be well defined. The peripheral areas are not well defined. The spectral component are additive (non-opponent) in the center and in the surround, except for the blue component which does not show opponency; rather, it shows one value, either excitation or inhibition in the center and surround.		
21 LNP/IRP		



Objectives: To study neural interactions and processing in a major sensory system. This study will provide insight on how information is organized and processed by a major neural plexus in preparation for transmittal to a distant neural plexus.

Methods Employed: Anesthetized and curarized intact experimental mammals such as the cat are placed in a special holder for stereotaxic placement of an interocular microelectrode to the retina. A modified maxwellian view optical stimulator permits precise light stimuli to be placed on the retina under direct visualization. Electrical responses are correlated with various parameters of the stimulus.

Major Findings: A study of the Spectral sensitivity of Retinal ganglion cells in the cat has been made using strong chromatic adaptation and spatial localization of the stimulus to the receptive center and/or periphery. The study has shown that three independent and chromatically distinct Cone receptor systems are present and converge on many if not all ganglion cells. Most show simple additivity of color components rather than opponency in the same region of the receptive field. A rare cell did show opponency however. Blue receptor input is found in a high percentage of ganglion cells, but not in opponency between center and periphery.

Significance to Biomedical Research and the Program of the Institute: This study indicates that the cat has a trichromatic visual system. The presence of three separate wavelength independent systems may be more broadly represented in mammalian species than previously believed.

Proposed Course: This project will be terminated with the publication of the manuscript listed below. Future work, using mammals will be carried out under Project Z01 NS 02339-05 LNP.

#### PUBLICATIONS

Crocker, Richard A., James Ringo, Myron L. Wolbarsht and Henry G. Wagner: "Cone Contributions to Cat Retinal Ganglion Cell Receptive Fields. J. Gen. Physiology 76: (1980) pp.763-785.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 NS 02152-07 LNP																
PERIOD COVERED October 1, 1980 to September 30 1981																		
TITLE OF PROJECT (80 characters or less) Neural Connections in the Retina																		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																		
<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 40%;">Henry G. Wagner</td> <td style="width: 10%;">Chief</td> <td style="width: 40%;">LNP, NINCDS</td> </tr> <tr> <td>OTHER:</td> <td>H. Kolb</td> <td>Research Biologist</td> <td>Univ. of Utah</td> </tr> <tr> <td></td> <td>R. Nelson</td> <td>Research Biologist</td> <td>NEI</td> </tr> <tr> <td></td> <td>A. Mariani</td> <td>Research Biologist</td> <td>NEI</td> </tr> </table>			PI:	Henry G. Wagner	Chief	LNP, NINCDS	OTHER:	H. Kolb	Research Biologist	Univ. of Utah		R. Nelson	Research Biologist	NEI		A. Mariani	Research Biologist	NEI
PI:	Henry G. Wagner	Chief	LNP, NINCDS															
OTHER:	H. Kolb	Research Biologist	Univ. of Utah															
	R. Nelson	Research Biologist	NEI															
	A. Mariani	Research Biologist	NEI															
COOPERATING UNITS (if any) Laboratory of Vision Research, NEI, Physiology Dept. Univ., of Utah, Salt Lake City, Utah.																		
LAB/BRANCH Laboratory of Neurophysiology																		
SECTION Section on Neuronal Interactions																		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																		
TOTAL MANYEARS: 0.3	PROFESSIONAL: 0.2	OTHER: 0.1																
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER																		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																		
SUMMARY OF WORK (200 words or less - underline keywords) Neuronal types contributing to the inner plexiform layer of the cat retina were studied using primarily light microscopy of <u>Golgi-impregnated retinal whole-mounts</u> . Cells have been characterized on morphological criteria that include <u>dendritic branching patterns</u> , dendritic tree sizes, cell body sizes and stratification of processes in the inner plexiform layer. Nine different types of <u>bipolar cells</u> , 22 different types of <u>amacrine cells</u> and 23 different types of <u>ganglion cell</u> were distinguished using one or more of these morphological criteria. The significance of the different morphological types of cells has been analyzed in relationship to the functional bisublamination of the cat inner plexiform layer.																		

23 LNP/IRP

## Project Description:

Objectives: To understand the neural circuitry of the vertebrate retina.

Methods Employed: 1. Light microscopy of Golgi-impregnated material. 2. Electron microscopy of Golgi-impregnated material. 3. Ultra-thin serial sectioning for electron microscopy. 4. Correlations with intracellular recordings and Procion marking of retinal neurons.

Major Findings: In a collaborative anatomical study of Golgi-impregnated monkey retinas, we have discovered a new type of horizontal cell with a distinctly different appearance from the hitherto described monkey horizontal cell type. The new type II horizontal cell has a profusion of fine, multibranched dendrites ending either in clusters or single terminals, and a short (100-300um length) convoluted axon which occasionally sprouts small clusters of terminals. In contrast, the type I horizontal cell has thick dendritic branches bearing large clusters of terminals and a stout axon which travels a direct course for 2mm before ending in a multibranched axon terminal. Type II horizontal cells have larger dendritic trees than type I cells in the foveal region but smaller dendritic trees than type I horizontal cells in peripheral retina. Golgi-EM of the new type II horizontal cells shows that the dendritic terminals contact cones and possibly some rods, while the groups of terminals on the short axon contact select cones. The photoreceptor connections of the type I monkey horizontal cells are already well documented. Calculations of space constants on the type II horizontal cell axon indicates that it probably behaves as a true axon conducting signals away from the cell body. It is hypothesized that the new HII cell contacts green and blue cones while the old HI cell may contact only red and green cones.

The morphology of physiologically identified neurons of the cat retinas has been determined by comparisons of HRP injected cells with Golgi-impregnated neurons. Cone bipolar cells that respond with a hyperpolarization to light prove to be flat cone bipolars, whereas cone bipolars responding with a depolarization to a flash of light are invaginating cone bipolars. Previously, we have shown that the dendritic branching of ganglion cells, either in the upper portion of the IPL where they receive flat cone bipolar input, or in the lower portion of the IPL where they receive invaginating cone bipolar input, determines whether the ganglion cells will be OFF center or ON center respectively. Thus, the ON/OFF center characteristics of direct cone bipolar/ganglion cell connected pathways in the retina, must originate before the bipolar ganglion cell synapses in the IPL: probably at the cone pedicle to cone bipolar synapses in the outer plexiform layer.

Several amacrine and ganglion cell types have also been studied with HRP after physiological investigation of their responses to light. Future EM analysis of the synaptic input to such marked cells is planned.

Golgi studies of carp and turtle retinas have been successful and correlations of the different cell types with physiologically identified cells will be possible. The turtle, for example, contains 8-10 bipolar types, 15-18 amacrine cell types and 18-20 ganglion cell types. We know that a large ganglion cell type branching high in the IPL is bipolar dominated whereas a large diffuse ganglion cell type is amacrine dominated from comparisons of these morphological findings with some physiological results in turtle retina. We hope in particular, to find morphological equivalents for orientation selective amacrine and ganglion cells in turtle retina by these methods.

Significance to Biomedical Research and the Program of the Institute:  
Studies of the structure of the retina will provide an understanding of the cells within the retina and will in all probability relate to neural circuitry elsewhere in the CNS. Many programs of this Institute are concerned with the physiology and marking of single retinal neurons and therefore knowing the morphology and connectivity of these neurons is essential for our further understanding of visual events.

Proposed Course  
The project will be terminated with the publication of a final report. Kolb, Helga, Ralph Nelson and Andrew Mariani. Amacrine Cells, Bipolar cells, and Ganglion cells of the Cat Retina. A Golgi Study (in press) - Vision Research - 1981.

PUBLICATIONS  
Kolb, Helga, Ralph Nelson and Andrew Mariani: Amacrine Cells, Bipolar Cells, and Ganglion cells of the Cat Retina. A Golgi Study (in press) - Vision Research - 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02339-04 LNP																					
PERIOD COVERED October 1, 1980 to September 30, 1981																							
TITLE OF PROJECT (80 characters or less) Neural Coding and Processing of Information in the Visual System																							
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																							
<table style="width: 100%; border: none;"> <tr> <td style="width: 30%;">PI H.G. Wagner</td> <td style="width: 40%;">Chief Section on Neuronal Interactions</td> <td style="width: 30%;">LNP, NINCDS</td> </tr> <tr> <td>K. Hara</td> <td>Special Expert</td> <td>LNP, NINCDS</td> </tr> <tr> <td>M.H. Wolbarsht</td> <td>Professor</td> <td>Duke Univ.</td> </tr> <tr> <td>E.F. MacNichol</td> <td>Director</td> <td>MSP-MBL</td> </tr> <tr> <td>A. Mariani</td> <td>Staff Fellow</td> <td>LVR-NEI</td> </tr> <tr> <td>M.A. Ali</td> <td>Professor</td> <td>Univ. of Montreal</td> </tr> <tr> <td>G.David Lange</td> <td>Assoc. Professor</td> <td>Scripps Institute</td> </tr> </table>			PI H.G. Wagner	Chief Section on Neuronal Interactions	LNP, NINCDS	K. Hara	Special Expert	LNP, NINCDS	M.H. Wolbarsht	Professor	Duke Univ.	E.F. MacNichol	Director	MSP-MBL	A. Mariani	Staff Fellow	LVR-NEI	M.A. Ali	Professor	Univ. of Montreal	G.David Lange	Assoc. Professor	Scripps Institute
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A. Mariani	Staff Fellow	LVR-NEI																					
M.A. Ali	Professor	Univ. of Montreal																					
G.David Lange	Assoc. Professor	Scripps Institute																					
COOPERATING UNITS (if any) Ophthalmology Department, Duke University, Durham, N.C.; Marine Biological Laboratory, Woods Hole, Mass.; Biology Department, University of Montreal, Canada; Scripps Institute of Oceanography, Calif.																							
LAB/BRANCH Laboratory of Neurophysiology																							
SECTION Section on Neuronal Interactions																							
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																							
TOTAL MANYEARS: 1.0	PROFESSIONAL: 0.9	OTHER: 0.1																					
CHECK APPROPRIATE BOX(ES)																							
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER																							
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																							
SUMMARY OF WORK (200 words or less - underline keywords) <p>             We have made a study of the sensitivity distribution profile of <u>receptive fields of retinal ganglion cells of carp</u>. The results lead us to believe that the sensitivity at threshold is highest at the center of the field and the sensitivity is progressively lower with increased distance from the center. We can differentiate two forms. One shows a relatively high sensitivity in the center and falls off rapidly at small radial distance from the center the second form has lower sensitivity at the center and decreases more gradually with distance from the center, "on" and "off" forms are found.           </p> <p>             The field diameter as revealed by profile plot is usually much less than that found to be the limit of <u>area-intensity reciprocity</u>. However we have noted reversible changes in both profile and limiting radius for area-intensity reciprocity for some ganglion cells. We interpret this to mean that the receptive field diameter is a function concept and is determined by the stimulus conditions.           </p>																							



Objectives: To study neural interactions and processing of stimulus information in the visual system. This study will provide insight on how information is organized and processed by a major neural plexus in preparation for transmission to the next neural plexus.

Methods Employed: Isolated, oxygenated retinæ of suitable fish such as the carp and goldfish are stimulated by light of known wavelength, intensity, duration and spatial configuration. Electrophysiological responses in ganglion cells or other neurons in the visual pathway are detected and analyzed with respect to the various parameters of the stimulus and their relationship to other neurons in the visual system.

Major Findings: It has been possible to sort out a number of spectrally distinct excitatory and inhibitory inputs to the ganglion cells of the carp. These inputs have spectral sensitivity distributions of complex form but initially, when isolated in very dim light show a  $\lambda$  max at 500 nm. More usually, exposure to moderate levels of background illumination produce spectral sensitivity curves with maxima at 600-650 nm. Stronger adapting backgrounds reveal  $\lambda$  max also at 500 nm and on occasion, at 450 nm. The spectral sensitivity curves may be narrow or broad. Broad curves invariably can be shown to be composed of two or more spectral components each having the same sign (on or off) but different  $\lambda$  max. Narrow curves occasionally show presence of more than one component and the additional component is of opposite sign. As many as three spectrally different components can often be found in the same cell. The variety of patterns found, suggests color vision in carp retinal ganglion cells is very complex.

A study of the sensitivity distribution over the receptive field of retinal ganglion cells in carp show a profile with a max at the center, and progressively lower sensitivity with increasing radius. Two forms of distribution were found. Area intensity reciprocity plots were made under various stimulus conditions. The relationship is accurate and a good description of the summation of inputs.

Significance to Biomedical Research and the Program of the Institute: This study will help understand the processing of neural information in the visual system.

Proposed Course of the Project: A major inquiry into the functional organization of the receptive fields of ganglion cells, amacrine and bipolar cells of vertebrate retinæ will be made.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02221-06 LNP
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less) Ionic Mechanisms of Phototransduction in Rods of the Vertebrate Retina		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: J. Schnapf Guest Worker LNP, NINCDS R.N. McBurney Visiting Scientist LNP, NINCDS		
COOPERATING UNITS (if any) Department of Physiology, University of Newcastle upon Tyne, England		
LAB/BRANCH Laboratory of Neurophysiology		
SECTION Section on Neuronal Interactions		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.1	PROFESSIONAL: 0	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) This project is completed and the results published in the two publications listed.		
28 LNP/IRP		

## Project Description:

Objectives: The principal objective of this study is to characterize the membrane mechanisms that are involved in the transduction of light stimulation into the electrical response of the photoreceptor.

Methods Employed: Suction pipette recordings of electrical potential and conductance were performed in the isolated retina using glass capillary microelectrodes on the outer segments of cones. The retina was mounted in a chamber which allowed superfusion of the photoreceptors with solutions of various ionic compositions. Dissection of the preparation and microelectrode positioning was accomplished using infrared visualization.

Major Findings: The membrane mechanisms by which light is transduced into electrical signals were investigated in photoreceptors. The rod photo-response results from two processes, a light modulated mechanism and a voltage and time dependent mechanism. Techniques for isolating each mechanism and the contribution of each to the photoresponse was studied. The dark potential and the photoresponse were recorded as a function of the concentration of external sodium, potassium and chloride ions as were the effects of 4 amino pyridine, cesium, and ouabain. From these measurements, it is concluded that in the dark, the rod membrane is 10 X more permeable to potassium than to sodium and that at peak of photoresponse, the sodium permeability is reduced by at least a factor of 10. We estimate that the cytoplasmic sodium and potassium concentrations are equal. These ionic gradients are maintained by active Na-K pumps.

Successful recordings were made of changes in the transmembrane current of the outer segments of cones in salamander and turtle. Calculated values of 0.012 pA per photo isomerization and conductance changes of 0.167 pS were obtained.

Significance to Biomedical Research and the Program of the Institute: This project has significance primarily at the basic research level. Much is known about the ionic mechanisms involved in propagation of electrical signals along the nerve axons and in synaptic transmission. To date an equally lucid description of the primary sensory transducer has not been presented which can account for the known observations. A comparison of the membrane mechanisms involved in the generation of the light response with the membrane properties of axons may provide fundamental insight into the basic mechanisms underlying neuron function.

Proposed Course of the Project: This project is terminated with the below publications.

Publications:

Julie L. Schnapf and R.N. McBurney: Light induced changes in membrane current in cone outer segments of tiger salamander and turtle. Nature 287: 239-241, 1980.

R.N. McBurney and J.L. Schnapf: The effect of light on membrane current recorded from cone outer segments. J. Physiol. 305: 72P, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 01659-13 LNP																
PERIOD COVERED October 1, 1980 to September 30, 1981																		
TITLE OF PROJECT (80 characters or less) Synaptic Contacts of Retinal Neurons																		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																		
<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 30%;">A. Lasansky</td> <td style="width: 30%;">Research Biologist</td> <td style="width: 30%;">LNP-NINCDS</td> </tr> <tr> <td></td> <td>Luigi Cervetto</td> <td>Senior Investigator</td> <td>LNP-CNR</td> </tr> <tr> <td></td> <td>Julie Lohr</td> <td>Technician</td> <td>LNP-NINCDS</td> </tr> <tr> <td></td> <td>W. Beane</td> <td>Technician</td> <td>LNP-NINCDS</td> </tr> </table>			PI:	A. Lasansky	Research Biologist	LNP-NINCDS		Luigi Cervetto	Senior Investigator	LNP-CNR		Julie Lohr	Technician	LNP-NINCDS		W. Beane	Technician	LNP-NINCDS
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COOPERATING UNITS (if any) Laboratory of Neurophysiology - C.N.R. Pisa Italy																		
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SUMMARY OF WORK (200 words or less - underline keywords) <u>Annular illumination decreases the input resistance of turtle cones and results in a depolarizing response when the intracellular concentration of Cl<sup>-</sup> is raised. Hyperpolarizing current blocks this synaptic response, while depolarizing pulses in darkness evoke potential and resistance changes similar to those associated with the surround effect. Furthermore, the response to annular illumination is transient and shows refractoriness. From these observations, it is suggested that the surround response of cones arises from the activation of a voltage- and time-dependent Cl<sup>-</sup> conductance.</u>																		



Objectives: To investigate the fine structure and function of synapses between retinal neurons.

Methods Employed: Electron microscopy combined with silver impregnations by the method of Golgi and intracellular injections of horseradish peroxidase. Electrical recordings with intracellular microelectrodes.

Major Findings: The previous work on retinal cones of the salamander indicated that annular illumination synaptically increases the chloride conductance of their membrane. It was also noted that hyperpolarizing current suppressed this response, an effect that suggested a voltage-sensitivity of the chloride channels involved in it. Further study of this property, however, required more stable recording conditions than those until now achieved with salamander cones. Therefore, the earlier observations have been repeated and extended in turtle cones, which proved to be a more favorable experimental material.

As for the earlier work on salamander cones, the responses of retinal cones of the turtle to steps of light on their surrounding area, extrinsic current, or a combination of both, were recorded intracellularly by means of micropipettes filled with 3M-potassium acetate or 3M-potassium chloride. When using 3M-potassium acetate, a dim annulus elicited a decrease in input resistance and a small depolarization (less than 2mV) that was not a consequence of the resistance loss. With 3M-potassium chloride, the decrease in membrane resistance outlasted the light step for a longer time and resulted in a depolarization of up to 15 mV in amplitude. With either electrolyte, hyperpolarizing pulses suppressed the change in input resistance induced by annular illumination. Depolarizing pulses lowered the membrane resistance; with Cl<sup>-</sup> electrodes, this led to a slowly rising depolarization that could outlast the pulse by more than 1 second. The response to steady illumination with the annulus was transient. High-frequency step illumination failed to be followed by the surround response according to a pattern compatible with refractoriness in a voltage-dependent process.

From these features, the surround response of cones seems likely to arise from the synaptic activation of a voltage- and time-dependent Cl<sup>-</sup> conductance, the existence of which is suggested by the responses to depolarizing current.

Significance to Biomedical Research and the Program of the Institute:

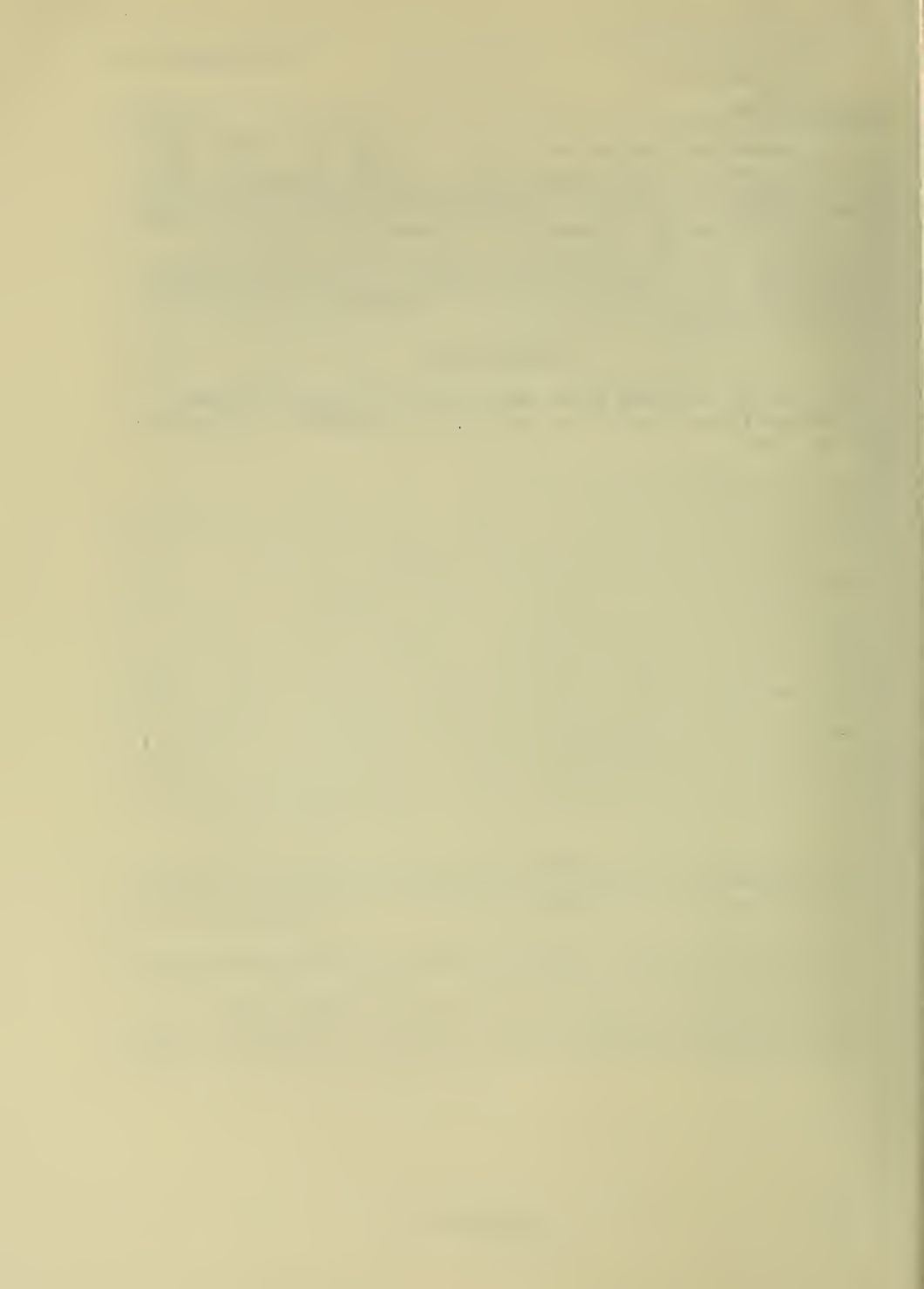
It is hoped that these observations will help in identifying the mechanisms of synaptic transmission between retinal neurons, and provide a better knowledge of the neuronal networks involved in the processing of visual information within the retina.

Proposed course

The observed surround responses of retinal cones are likely to be due to synaptic feedback from horizontal cells. Retinal rods, on the other hand, are generally thought not to receive such a feedback or any other chemical synaptic input, but no detailed experimental study of this question has been reported. Consequently, an attempt will be made to settle this matter and to establish what differences, if any, exist in this regard between the two types of retinal receptors, by using the methods outlined above to analyse the responses of salamander rods to illumination of their surround.

PUBLICATIONS

A. Lasansky. Synaptic action mediating cone responses to annular illumination in the retina of the larval tiger salamander. J. Physiol. 310: 205, 1981









## ANNUAL REPORT

October 1, 1980 through September 30, 1981

### Laboratory of Biophysics

National Institute of Neurological and Communicative Disorders and Stroke

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National Institute of Neurological and Communicative  
Disorders and Stroke  
Laboratory of Biophysics  
William J. Adelman, Jr., PhD, Chief

## INTRODUCTION

The research program of the Laboratory of Biophysics is concerned with investigating molecular and cellular mechanisms responsible for excitation, membrane potentials, the generation of the nerve impulse, synaptic activity, the biophysical basis for the functioning of simple nervous systems, and the cellular basis for such integrative neural functions as behavior and learning. The laboratory makes wide use of physical and chemical techniques, on-line and off-line digital computers and a variety of applied mathematical methods. The laboratory is composed of two units. One of these units operates on a year-round basis at the Marine Biological Laboratory in Woods Hole, Mass. The Woods Hole Unit is composed of 2 sections: the Section on Neural Membranes and the Section on Neural Systems. The Bethesda unit of the laboratory is made up of the Section on Molecular Biophysics.

## WOODS HOLE UNIT OF THE LABORATORY OF BIOPHYSICS:

### Section on Neural Membranes.

The Section on Neural Membranes uses modern electrophysiological, electron optical, mathematical biophysical, and computer science techniques to investigate the function and structure of neural cells and tissues at limits approaching the molecular level. The general approach is to examine mechanisms that universally and fundamentally underlie all neural function. Emphasis is placed on membrane ionic channel structure and function. Model systems are derived, tested and used to simulate neuronal function under a variety of natural and experimental conditions. Subcellular structures supportive of axoplasmic transport and membrane ionic channel formation are sought. The physical mechanisms involving the structures of muscle and nerve responsible for contraction and mechanotransduction are probed and these are related to both the biochemical and structural elements underlying these mechanisms.

The project on voltage clamp and impedance measurements in collaboration with the University of New Hampshire showed that aphantoxin (ATX), an agent contained in the blue-green fresh-water alga Aphanizomenon flos-aquae, blocks the squid axon membrane sodium conductance. The blocking effect of ATX appears to be completely reversible and has no effect on the potassium conductance. Channel blockage by ATX follows a dose-response curve representative of 1:1 stoichiometry similar to the action of tetrodotoxin and saxitoxin. The potential utility of ATX as a "tool" for studying membrane systems should be of importance to neurobiology, irrespective of the public health problems posed by world-wide blooms of the toxic Aphanizomenon algae in fresh water lakes.

In collaboration with Emory University and Rockefeller University, this same project has continued to examine the quasilinear behavior of excitable membranes

as described by the squid axon's membrane impedance or admittance. On theoretical grounds it was predicted that at some steady membrane potential,  $V_0$ , and some perturbation frequency,  $f$ , the real and imaginary parts of the admittance of the squid axon membrane are zero; this means that the amplitude of the admittance (the modulus) is zero at these values of  $V_0$  and  $f$ .

Upon voltage clamping the squid axon, it was shown that the membrane admittance goes through a minimum at different holding potentials,  $V_0$ , and at some particular frequency. However, at no value of membrane potential or perturbation frequency, however carefully one attempts to explore these variables, does the amplitude of the admittance go to zero by this method. When a frequency is used which gives the minimum current amplitude, the current is nearly in phase with the voltage (zero phase condition). At lower frequencies current leads voltage (an inductive response); at higher frequencies voltage leads current (a capacitive response). At the zero phase frequency, higher order current components, particularly a  $2f$  component, are observed as are predicted theoretically.

The effect of TTX, a sodium channel blocker, on the current at the zero phase condition was measured. TTX increased the first order current because at zero phase condition the Na current normally subtracts from other currents to produce the minimum. TTX decreases the second order current and the ratio  $|I_2|/|I_1|$  becomes smaller than normal. In unblocked axons the ratio  $|I_2|/|I_1|$  is largest near the zero phase frequency.

The conclusion drawn from this work is that, although the experimental membrane is mostly nonlinear for the zero phase condition and for 2 to 6 mV depolarization, it is less nonlinear than the theoretical membrane derived from the Hodgkin-Huxley model. Explanations for this discrepancy await further analysis.

The project on function and structure of ionic channels (ion interactions and gating mechanisms) has moved ahead on several fronts. A study was made deriving both theoretically and experimentally the gating kinetics for a stochastic single channel from the kinetics of conductance changes determined from conventional macroscopic voltage clamp data. This study was done in collaboration with the University of Minnesota.

Another study done in collaboration with Minnesota examined squid axon potassium channel gating rates as functions of calcium and potassium concentrations. With near normal internal and external monovalent ionic concentrations, the rise-time of the potassium conductance under voltage clamp increases when the external calcium concentration is raised above normal. However, the rise-time values were shown to be inversely related to external calcium concentration when K concentrations are relatively low internally and relatively high externally. Making use of a surface charge screening hypothesis, an explanation has been proposed as based on a reciprocal interaction between a conducting channel ion and channel gate-charges.

Another aspect of this project involved a collaborative study with the Institute for Physical Sciences and Technology of the University of Maryland. The study derived a model for describing the influence of previous electrical history on conductance changes in a nerve membrane. The model proposes that the rate constants governing transitions of gating particles between closed and open states are dependent not only upon membrane potential, but also on time. These rate constants should not instantaneously follow a change in membrane potential.

A physical picture was proposed based upon a partial screening of the electric field in the vicinity of the gating particles by adjacent lipid molecules.

This same collaboration produced a study of the activation of potassium ion conductance in squid axons by depolarizing voltage steps preceded by conditioning hyperpolarizing voltages. This procedure results in a delay in the turn-on of the potassium conductance. While previous studies have shown that the control conductance kinetics superpose with the delayed kinetics when they are translated along the time axis by an amount equal to the delay (Cole and Moore, 1960), the present work has found that the degree of superposition depends upon experimental protocol. The kinetics superpose almost exactly for modest test depolarizations whereas they clearly fail to superpose for more positive levels of membrane depolarization. These results were modeled by incorporating a time dependence into the rate constant of activation of potassium channel gates in the Hodgkin-Huxley model of potassium ion conductance.

In a study in collaboration with the University of Texas Medical Branch in Galveston, voltage clamped perfused squid axons were studied under conditions of altered, macroscopic solution viscosity. Sucrose added internally or on both sides of the membrane caused slowing of the kinetics and decreases in the peak and steady state amplitudes of both sodium and potassium conductances. Sucrose effects on sodium ionic currents were paralleled by effects on sodium gating currents. In contrast, addition of 12.5% Ficoll (MW = 400K; macroscopic viscosity equivalent to 1.5 M sucrose) to the internal solution had little effect on  $g_{Na}$  and addition to both sides of the axon led to a 1.35 fold increase in  $\tau_m$  with no change in  $\tau_h$ . Conductivity measurements showed that Ficoll had little effect on microscopic viscosity. This differential effect of sucrose and Ficoll suggests that changes in microscopic viscosity reflected by changes in ionic mobility and water activity may have a direct effect on nerve membrane channel gating behavior.

The project concerned with subcellular and extracellular structure associated with nerve and muscle has developed a method for characterization of periodic structure in subcellular macromolecular arrays by computer processing of scanning transmission (STEM) video signals. Application of Fourier analytical methods to the video line signals comprising the picture raster in scanning transmission electron microscopy (STEM) represents a convenient and objective method for characterization of the periodic structure inherent in many subcellular macromolecular arrays. Among the model systems explored are the network structure in thin sections of embedded tropomyosin crystals, cross sections of myelin sheath and longitudinal sections of squid mantle muscle fibers. In the simplest method, a prominent specimen axis is aligned along or across the line scanning direction in single line mode and the image focused using y-mode presentation. This single line video signal is recorded repetitively (typically 128 or 256 lines) on a digital signal processor to reduce inherent noise and transferred to a computer for analysis. In most instances, the periodicity visible in the full STEM picture can be observed and directly plotted using a cursor on the single line y-mode signal. Examination of the forward Fourier transform of this trace (visualized as a power spectrum) shows a frequency peak corresponding to this spacing together with extraneous lower and higher frequency peaks arising from specimen background and other noise. Often, several orders of the fundamental are seen, depending on the nature of the specimen. Background noise arising from specimen irregularities caused by sectioning or staining inhomogeneities can be eliminated by removing the lower and/or higher frequency components before carrying out a reverse Fourier transformation. This Fourier filtering can be applied to whole image data as



well.

This project has made use of the Fourier analysis method and other analytical methods to characterize neuroplasmic order in vertebrate and invertebrate axons. Evidence for an ordered neuroplasmic lattice or network in Loligo and Hermisenda axons ( $0.5\ \mu\text{m}$ ) sections has been extended by using the electronic advantages of STEM and by taking advantage of the low chromatic aberration TEM characteristics of the Philips EM400. The neuroplasmic lattice consists primarily of neurofilaments together with their periodically arrayed side projections ( $40\text{--}45\ \text{nm}$  apart) which appear to act as cross-bridges. The lattice often includes neurotubules and other filamentous elements. This lattice structure spatial continuity appears to account for the gel-like character of axoplasm and its anomalously low optical anisotropy. An essentially identical neuroplasmic lattice structure and cross-bridge spacing is observed in Bufo peripheral axons both in internodal regions and in constricted zones associated with nodes of Ranvier and Schmidt-Lanterman clefts. In zones of the latter type, the neurofilament packing density often exceeds  $1000/\mu\text{m}^2$ , a value approaching that ( $1283/\mu\text{m}^2$ ) for hexagonal close packing of elements with a lateral spacing of  $30\ \text{nm}$ . This closer packing of neurofilaments appears to allow better preservation of lattice order during specimen preparation. Imaging of the neuroplasmic lattice in longitudinal sections by STEM has allowed preliminary characterization in terms of cross-bridge spacing by using Fourier analytical methods. In all cases examined, the neuroplasmic lattice showed an apparent longitudinal spacing of  $40\text{--}45\ \text{nm}$ , in good agreement with TEM stereo and autocorrelative findings. The STEM and TEM results both suggest that the cross-bridges might be related to a larger unit cell by screw axis symmetry.

The project investigating electro-mechanical coupled interactions in excitable tissues has examined in detail membrane potential changes during stretch in squid giant axons. The characteristic response to sudden stretch was an initial depolarization which slightly lagged stretch, decaying within two msec of termination of lengthening and usually ending in a brief wave of hyperpolarization. Stretches applied to axons in ASW compared to ASW containing TTX revealed two components of the initial depolarization. The primary rapid component was correlated with rate and amplitude of stretch and was not abolished by TTX. The slower, secondary component was abolished by TTX and ASW solutions in which choline replaced  $\text{Na}^+$ . The rapid repolarization seen at the end of lengthening could not be delayed or avoided by maintaining the stretch. The period of hyperpolarization was increased in amplitude and duration by increasing stretch amplitude. Some axons showed sequentially increasing electrical responses to each of several identical stretches applied at five minute intervals. This ultimately led to action potential generation. The mechanically evoked action potentials showed time course, amplitude and threshold membrane potential similar to those evoked by current pulses. These results suggest that the primary depolarizing response to sudden membrane strain activates the conventional ionic channels involved in regenerative discharge phenomena.

#### Section on Neural Systems.

The objective of the Section on Neural Systems is to study the mechanisms by which simple neural networks process information with particular emphasis on mechanisms of learning. Information processing involving sensory transduction, synaptic interactions, intersensory communication, conditioning paradigms, membrane and synaptic modification, developmental stages and biochemical mechanisms is of interest. Several marine specimens are used as experimental prepara-

tions. The principle preparation is the nudibranch mollusc, Hermissenda crassicornis. Methods of study include electrophysiologic, biochemical, electron microscopic, behavioral and developmental techniques. The following are highlights of the experimental program of the section during this reporting period.

Membrane changes measured by voltage clamp techniques, intrinsic to single identified neurons within the Hermissenda nervous system, were found to predict the level of performance of conditioned intact animals. This predictive correlation of single neuronal channel modification provides strong evidence that these neuronal transformations initiate a sequence or cascade of neural network changes which cause associative learning.

The membrane changes have been characterized under voltage clamp. A voltage-dependent potassium current undergoes long-lasting inactivation. This inactivation is a consequence of prolonged, accumulated membrane depolarization which occurs specifically during conditioning of the animals. A comprehensive characterization of these voltage-dependent  $K^+$  currents has been accomplished to now permit mathematical description and modelling.

The biochemical basis for inactivation of this voltage-dependent potassium current is now being established. It was found that the catalytic subunit of protein kinase, when iontophoresed into the neuron believed to be the primary site for storage of the stimulus association, induces similar membrane changes, i.e., inactivation of dark  $K^+$  currents, to those which occur during learning. This finding, together with the identification of enhanced phosphorylation of individual neuronal proteins during learning, suggests that the potassium channel changes arise from shifts of intracellular cyclic nucleotide metabolism. These shifts are also now being studied in relation to methylation and protein synthesis within the neurons of interest.

The cascade of neural network changes during associative learning are now being assessed with the 2-deoxyglucose technique. With this technique, it has become possible to define complete sensory pathways as they respond to preferred natural stimuli such as light, gravity and food substances.

Success was also achieved in reducing the associative behavioral changes into components which can be related to activity of individual motor neurons. Intracellular penetration and stimulation of these neurons is now revealing how these neurons respond to changes of synaptic input during learning to cause changes of the animals' movements.

#### BETHESDA UNIT OF LB:

##### Section on Molecular Biophysics.

The goals of the Section on Molecular Biophysics are to determine the mechanisms of action of membrane ionic channels and of drugs that interact with these channels.

Previous studies on cultured rat and chick skeletal muscle demonstrated the single-channel currents induced in cholinergic channels by the agonists acetylcholine, suberyldicholine and carbamylcholine. The focus of these studies was on the elucidation of gating kinetics at the single-channel level and a search for kinetic features which might be modified during cell development or synaptic-

channel aggregation. The single-channel studies have been extended to satellite cells of tissue-cultured human muscle. Human muscle confirmed the main features of other mammalian muscle, and a more systematic study was made of how channel kinetics are modified during cell development.

One outgrowth of the muscle studies was the demonstration in embryonic rat and human muscle that the muscle poison curare (d-tubocurarine), which is a classical postsynaptic blocker, actually activates ionic channels (for short times,  $\sim 200 \mu\text{s}$ ) in early embryonic states. From the results on this agonist action, an attempt was initiated to explain the action of partial agonists as cholinergic agents which have relatively long residence times at membrane sites, but can only effect channel openings in a transient, flickering manner. To date, success has been in measuring the single-channel currents induced by the partial agonists choline, decamethonium and succinylcholine.

One of the reasons suggested for why the curare activation changes during development is that the fatty acid composition changes and that this, in turn, modifies the gating conformation changes. At present, this notion is being tested by recording channel currents from cells grown in media enriched with different fatty acids. In a parallel effort, attempts are being made to dope muscle membrane with spin-labelled fatty acids to act as probes of membrane fluidity in developing cells.

The single-channel studies have also been extended to central nervous system neurons. It has been possible to record the channels induced in mouse spinal cord neurons by the inhibitory transmitter GABA and by the mushroom toxin, muscimol. Attempts were made to record single serotonin channels in neuroblastoma cells. These have proven difficult to record, but a thorough voltage-clamp study of the serotonin response is nearly complete.

Another new application of the patch electrode method begun in the past year is the attempt to record single channels in the crayfish stretch neuron in response to mechanical stimulation of the attached muscle cell.

In order to analyze single-channel records in general and those with low signal-to-noise ratio in particular, it is important to use objective criteria to establish the presence of single channels. A method for the analysis of noisy experimental records of square waves resulting from the opening and closing of single channels in a membrane has been developed, using statistical detection theory. A computer program has been written to simulate such signals and to test the detection method by comparison of the reconstructed signal with the original one, with satisfactory results. The more difficult problem of detection of a noisy signal which has been distorted by a low-pass filter is now being studied.

Another approach to determine molecular mechanisms for the behavior of ionic channels is to develop molecular models of the channels or of significant portions of them. In particular, this approach has been utilized for a portion of the postsynaptic acetylcholine channel. The development of a molecular model was based on the known amino acid sequence. Then methods were developed for predicting secondary structure and for determining the way in which alpha helices can stack together to form a barrier between water and the lipid phase of the membrane. Utilizing these methods, a three-dimensional molecular model was determined. This model has one conformation that includes a region through which ions can easily pass and another conformation which prevents ions from passing.



In line with the Section's objective of determining mechanisms for the action of ionic channels, we have begun a set of experiments employing spectroscopic probes of channel conformation change. Cholinergic channels isolated from Torpedo electroplax have been labelled with paramagnetic and fluorescent probes. The channels are then treated with agonists and antagonists and a slow conformation change can be monitored. At present, whether this conformation change can be identified with channel desensitization is under test.

Two classes of drugs that affect sodium channels have been studied. One class consists of drugs, such as local anesthetics and yohimbine, that block sodium channels. The other class consists of drugs, such as batrachotoxin and veratridine, that increase the probability of opening sodium channels.

In one set of experiments, the hypothesis that all local anesthetics have a common receptor was tested - a hypothesis that has been put forward in the literature. If this hypothesis is correct, two different local anesthetics should be competitive inhibitors. The preparation used was neuroblastoma, and the drugs tested were benzocaine (which is uncharged) and QX-572 (a charged derivative of lidocaine). It was found that these drugs are not competitive inhibitors of sodium channels; they are cooperative noncompetitive inhibitors. This result is consistent with each drug having a separate site of action for blocking sodium channels.

The cooperative nature of the interaction of these two local anesthetics results in a synergism, which may be clinically useful. According, calculations were made of the clinical advantages that might be obtained by using two drugs (such as local anesthetics) that are cooperative noncompetitive inhibitors. On the basis of several reasonable assumptions, it was found that such two-drug therapy would reduce side effects below the level of one-drug therapy, and that the magnitude of the reduction could be significant.

Batrachotoxin, a channel-opening drug, was also examined in neuroblastoma. Preliminary results indicate that the channel-opening property of batrachotoxin is based on a shift of the voltage-dependent parameters of sodium channels along the voltage axis. It is planned to test whether the result also applies to other channel-opening drugs.

Studies on the distribution and kinetics of dielectric polarization in the membrane of the giant axon of the squid have continued in collaboration with the University of California at Los Angeles. Comparisons between time and frequency domain results of gating current measurements have been analyzed in terms of four- and eight-state simplified models and were found to be in excellent agreement.

Measurements of membrane complex capacitance were extended to lower frequencies (200 Hz to 10 kHz) at membrane potentials where the gating currents were shown to be small.

Considerable insight into the origin of slow inactivation of sodium conductance has been gained by the studies of long-term inactivation of gating currents which were analyzed in terms of the simplified eight-state model.

It has been known for many years that some drugs show use-dependence inhibition on excitable membranes, i.e., the extent of inhibition is highly dependent upon the recent history, or "use", of the sodium channels. Drugs, which exhibit

use-dependent inhibition, also inhibit the sodium conductance when there has been no recent "use". This latter effect is known as the tonic effect, in contrast to the use-dependent effect. One drug which the Section has studied extensively for its use-dependent effects on the squid giant axon is yohimbine. In order to understand the mechanism of these two effects, a study was begun of the drug's structure-activity-relationships. Yohimbine possesses three asymmetric carbon atoms giving rise to the normal, pseudo, allo, and epiallo configurations. There are reactive groups on carbon positions 16 and 17. These studies indicate using nine analogs of yohimbine that both normal and allo configurations are active and indistinguishable. Altering the reactive groups on positions 16 and 17 only had some quantitative effect. The tentative conclusion is that the basic requirement for the use-dependent and tonic effects of yohimbine may reside in the yohimbine skeleton.

Since sea water, either natural or artificial, has been used as an external medium for the squid giant axon, it was decided to measure the calcium and potassium activities of the hemolymph of the squid. The activity ratio between the hemolymph and sea water was 0.99 for calcium and 1.26 for potassium. It has been shown by others that, in order for the excised squid axon to be in a steady state, the concentrations of the natural 10 mM calcium sea water has to be lowered to 3 mM calcium, corresponding to a ratio of 0.3. Perhaps, this difference in the ratio is due to a change in the active transport or permeability for the calcium in the excised nerve. For potassium, concentration ratios have been reported to be 1.34 to 2.22. It was found that the potassium ratio could be increased significantly by a delay in obtaining the hemolymph sample, presumably due to potassium leakage from the cells into the hemolymph. Such a leakage could explain the higher potassium concentration ratios obtained by some investigators.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 01950-10 LB																																							
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TITLE OF PROJECT (80 characters or less)  Excitable Membrane Characteristics: Voltage Clamp and Impedance Measurements.																																									
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COOPERATING UNITS (if any) Marine Biological Laboratory, Woods Hole, MA 02543; Univ. of Minnesota; Rockefeller Univ.; Emory Univ.; Univ. of New Hampshire; McGill Univ.																																									
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TOTAL MANYEARS: 4.0	PROFESSIONAL: 3.8	OTHER: 0.2																																							
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SUMMARY OF WORK (200 words or less - underline keywords) <p>             The general aim of this project has been to study excitable membrane characteristics by a variety of physical methods. One aspect has been to improve <u>electrical measurements of excitable membrane characteristics</u> consistent with <u>physical and chemical methods</u> for the study of nerve and muscle membrane ionic channels. Two major approaches are used. The first involves the development of methods for <u>analysis of ionic channel admittances and/or conductances</u> by means of <u>voltage clamp techniques</u>. Programs for carrying out this analysis are developed. The second approach involves analysis of excitable membrane characteristics by means of <u>frequency analysis of admittance measurements on giant axons</u>. An investigation of effects of polarizations for comparison with ion conduction models is carried out. <u>Voltage and current clamp experiments</u> are employed to characterize the ionic currents underlying excitability in <u>squid giant axons</u> and <u>chick embryonic heart cells</u>. The contributions of the various currents to <u>voltage oscillations, pacemaker potentials and action potentials</u> are determined by <u>computer simulations</u> based on the voltage clamp measurements.           </p>																																									

Project Description:Objectives.

- 1) To study the blocking effect on nerve membranes of a new neurotoxin, Aphantoxin (ATX), derived from a blue-green fresh-water alga, Aphanizomenon flos-aquae. To examine the toxin's site of action, its specificity and reversibility, and to determine its dose-response relation and stoichiometry.
- 2) To study the effect of surface charges, their local distributions and strong electric fields produced locally by conducting ions within membrane channels on the potassium channel gating mechanism in axons.
- 3) To determine the excitable axonal membrane admittance by studying linear and nonlinear conductance components in the frequency domain. To relate this admittance to conventionally determined macroscopic membrane currents and to currents flowing through fluctuating microscopic single channels.
- 4) To determine the similarities and differences of ionic channels in nerve and heart. To determine the pacemaker currents which underlie excitability of spontaneously beating embryonic heart cells. To model the ionic currents using mathematical formulations similar to the Hodgkin-Huxley model. These results are then used to simulate voltage changes in response to current stimuli or perturbations in ionic environment.

Methods Employed.

1) Toxic algal samples were collected from Kezar Lake, North Sutton, New Hampshire during a bloom essentially unialgal with A. flos-aquae using a DeLaval separator. Samples were frozen as a thick sludge. The cells were broken by several thawing-freezing cycles and the cell debris was separated by centrifugation. The supernatant was passed through 10,000 Dalton, and subsequently 500 Dalton Amicon filters, resulting in a clear filtrate containing ATX, salts, amino acids and other low molecular weight materials. The filtrate was dried and then reconstituted with distilled water. A standard mouse assay commonly employed for paralytic shellfish poison (STX) in marine bivalves was used as a potency check with the result that the reconstituted solution led to survival times in mice between 4 to 6 minutes upon i.p. injection. On the basis of this result, the reconstituted solution is the toxic equivalent of 0.42 micrograms of saxitoxin/ml. Artificial seawater solutions containing STX-equivalent concentrations of 100, 30, 10, 3 and 1 nanomolar ATX were prepared for testing. Squid axons, initially bathed in toxin-free artificial seawater (ASW), were voltage clamped to a series of depolarizing membrane potentials, and the corresponding membrane currents were recorded. The external bathing solution was then changed to one of the ATX/ASW solutions. A test depolarizing pulse of 60 mV (from a fixed holding potential, typically -60 mV) was used during brief periods of voltage clamp to determine when the membrane current reached a new steady value. The axons were then clamped to the same set of voltage pulses used initially and a set of membrane currents were recorded. The axons were then returned to ASW and recovery from toxin-induced block was followed until steady values of the test membrane current were achieved. The axons were voltage clamped again to the same series of potentials used initially. In some experiments this procedure was repeated several times using the same axon.

2) Squid giant axons were internally perfused with a variety of solutions in which potassium concentration and ionic strength were varied. External concentrations of potassium and calcium ions were also varied. These axons were voltage clamped and isochronal, chord and instantaneous potassium conductances were measured. Surface charge theory was applied to the data to account for voltage shifts in  $g_K$  conductance kinetics brought about by the solution changes.

3) Space clamped giant axons were voltage clamped near rest with the wave form  $V(t) = V + V_0 \cos(2\pi f_0 t)$ . Fourier transformations of the membrane currents were performed. Power spectra were plotted and analyzed and current frequency peak determined.

4) The excitable properties of squid giant axons are studied with the aid of voltage clamp methodology. When desirable, the internal composition of the axon may be manipulated through the use of an internal perfusion system. Extensive use is made of digital techniques for control of the experiment and data analysis. The excitable properties of chick embryonic heart cells are studied with a two microelectrode voltage clamp of small clusters, or aggregates, of electrically coupled cells. When desirable, the ionic components of the tissue culture medium bathing the aggregates can be modified by a perfusion system. Membrane currents are recorded on analog FM tape for off-line analysis.

### Major Findings.

1) Aphantoxin (ATX), an agent contained in the blue-green fresh-water alga *Aphanizomenon flos-aquae*, blocks the sodium conductance of the squid axon membrane with no effect on the potassium conductance. Channel blockage by ATX follows a dose-response curve representative of 1:1 stoichiometry similar to the action of tetrodotoxin (TTX) and saxitoxin (STX). The blocking effect of ATX appears to be completely reversible.

2) In a series of voltage clamp experiments whose protocol involved a series of calcium concentrations (2, 10, 40, 100 mM) externally, each coupled with series of internal ionic strengths (863, 575, 288 mM) in association with bulk potassium ion concentration gradients ( $[K]_i/[K]_e = 450/5, 300/10, 300/50, 150/50$ , all mM), it was observed that the voltage shifts of the time constants for delayed rectification which normally accompany a  $[Ca]_e$ -series are a strong function of the K-gradients. These shifts are +6 to 9 mV for an e-fold reduction of  $[Ca]_e$  for 450/5 and 300/10; almost no shifts for 300/50; and -6 to -9 mV/e-fold reduction of  $[Ca]_e$  for 150/50. The time constants are independent of  $[K]_i/[K]_e$  for bivalent cationic concentrations found in normal sea water. We conclude that electric field changes at the gating voltage sensor cannot be accounted for simply by the screening of fixed surface charge in conjunction with calcium binding. It seems likely that the local field, due to conducting ions in preferential positions within closed channels, is equally important. The data were further evaluated in conjunction with the Grahame equation and the behavior of instantaneous conductance to determine the distribution of fixed charge near the channel at the internal and external membrane surfaces. There appears to be a negligible amount of charge on the internal surface both near the "pore" opening and in a position to influence the voltage sensor for the channel gate. Further, there appears to be little charge near the external "pore" opening, but there is charge with relatively strong calcium binding properties (association constant =  $30M^{-1}$ )

in a position to influence the channel gating "machinery".

3) Fourier transformations of voltage clamped membrane currents, in response to sinusoidal membrane voltages, revealed sinusoidal components at  $f_0$ ,  $2f_0$ , etc. For  $V_0$  near 1 mV and  $f_0$  near the resonant frequency of the membrane, the amplitude of the  $2f_0$  component increased with depolarization, decreased with TTX and varied approximately as  $V_0^2$ . The behavior of the  $f_0$  and  $2f_0$  components was in qualitative agreement with the Hodgkin-Huxley equations; however, the null amplitude of the  $f_0$  component, predicted by the HH-equations to occur at particular values of  $V$  and  $f_0$ , was not found. Both  $f_0$  and  $2f_0$  components for an axon clamped at rest were found. The larger peak at 68 Hz ( $f_0$ ) corresponded to 113 nA, the smaller ( $2f_0$ ) peak at 136 Hz corresponded to 2.9 nA. Decreasing  $f_0$  to 20 Hz increased the  $2f_0$  component; increasing  $f_0$  to 200 Hz decreased it. Higher order components were also studied.

4) We have fully characterized a potassium ion, time-dependent current in embryonic heart cells which is partly responsible for the spontaneous activity of these preparations under normal tissue culture medium conditions. The current is activated in the -90 to -60 mV range of membrane potential. The peak time constant for activation of the current is approximately 1 sec, which occurs at -75 mV. The fully activated, i.e., instantaneous, current-voltage relation inwardly rectifies. Under certain conditions, the membrane potential oscillates prior to a beat in a manner qualitatively similar to the behavior of squid giant axons in artificial seawater containing lower than normal concentrations of calcium ions. The time scale of oscillations is several milliseconds in nerve, whereas it is several seconds in heart. We have successfully simulated our results from heart cells using mathematical models of our voltage clamp measurements.

#### Publications:

DeFelice, L.J., Adelman, W.J., Jr., Clapham, D. and Mauro, A.: Second order admittance in squid axon. In Adelman, W.J., Jr. and Goldman, D.E. (Eds.): The Biophysical Approach to Excitable Systems. New York, Plenum Publishing, 1981, pp. 37-63.

Adelman, W.J., Jr., Fohlmeister, J.F., Sasner, J.J., Jr. and Ikawa, M.: Sodium channels blocked by Aphantoxin obtained from the blue-green alga, Aphanizomenon flos-aquae. Toxicon. (In press).

Clay, J.R. and Shrier, A.: The mechanisms underlying pacemaker oscillations in chick embryonic heart cells. J. Physiol. (London). (In press).

Adelman, W.J., Jr. and Goldman, D.E. (Eds.): The Biophysical Approach to Excitable Systems. New York, Plenum Publishing, 1981, 258 pp.

Fohlmeister, J.F. and Adelman, W.J., Jr.: Anomalous potassium channel gating rates as functions of calcium and potassium ion concentrations. Biophys. J. (In press).



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02087-08 LB
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less) Function and Structure of Ionic Channels: Ion Interactions and Gating Mechanisms.		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: W. J. Adelman, Jr. Other: R. J. French J. J. Shoukimas J. R. Clay L. J. DeFelice M. F. Shlesinger J. F. Fohlmeister	Chief Assistant Professor Staff Fellow Staff Fellow IPA Fellow Research Scientist Assistant Professor	LB NINCDS Univ. of Maryland LB NINCDS LB NINCDS LB NINCDS Univ. of Maryland Univ. of Minn.
COOPERATING UNITS (if any) Marine Biological Laboratory, Woods Hole, MA 02543; University of Maryland; University of Minnesota		
LAB/BRANCH Laboratory of Biophysics, IRP		
SECTION Section on Neural Membranes (located at MBL, Woods Hole, MA 02543)		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 3.3	PROFESSIONAL: 3.3	OTHER: 0.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Voltage clamp experiments are employed to determine functional and structural characteristics of <u>ionic channels</u> in the squid <u>giant axon</u> . Information concerning these characteristics of the ionic channels is gained by studying the interaction of ions which <u>block</u> the passage of normal charge carriers and by studying the effect of <u>voltage</u> upon the opening and closing (" <u>gating</u> ") of channels. Computer simulations are performed of discrete openings and closings of single potassium and sodium <u>ionic channels</u> in nerve and heart using results from probability theory and a random number generator. The <u>gating kinetics</u> of <u>stochastic</u> single K-channels are related to the kinetics of conventionally defined <u>conductances</u> .		



Project Description:Objectives.

- 1) To determine whether kinetics of K-channel block are altered by increased solution viscosity.
- 2) To characterize the effects of microviscosity upon macro- and microscopic properties of ionic channels.
- 3) To determine the effects on potassium currents of large concentrations of external cesium and rubidium ions. To determine the degree of superposition after time translation of Cole-Moore kinetics of potassium channels.
- 4) To determine the behavior of single ionic channels in voltage and current clamp conditions based upon various different kinetic schemes for channel gating.
- 5) To relate the gating kinetics of single potassium channels to the kinetics of conventionally defined macroscopic conductances.

Methods Employed.

1,2,3) The excitable properties of squid giant axon are studied with the aid of voltage clamp methodology. When desirable, the internal composition of the axon may be manipulated through the use of an internal perfusion system. Extensive use is made of digital techniques for control of the experiment and data analysis.

4) A computer program is used which determines the durations of a sequence of random opening and closings of a single channel during various conditions, such as a voltage clamp step or an action potential. The probability function required for this procedure is determined from theoretical considerations. For example, a channel with a single gate can be described by  $[C] \xrightleftharpoons[\beta]{\alpha} [O]$ , where  $[C]$  represents the closed state,  $[O]$  represents the open state, and  $\alpha(\beta)$  represent the rate constant for opening (closing). In steady voltage clamp conditions, the probability  $P_o(T)$  that any single open time does not exceed the time,  $T$ , is given by  $1 - e^{-\beta T}$ . Similarly, the probability,  $P_c(T)$ , that any single closed time does not exceed  $T$  is given by  $1 - e^{-\alpha T}$ . These results may be generated for any other kinetic model.

5) Although it is impossible with conventional voltage clamp techniques to determine the absolute probabilities for a single channel to be in the conducting state, the relative probabilities can be determined provided the instantaneous driving force is measured. This information is derived from sets of instantaneous current-voltage relationships, but is not contained in the chord conductance. Mathematical relationships connecting the single channel conductance probability with conventionally measured parameters are derived.

### Major Findings.

1) Tektrakis (tetraethanolammonium ion) blocks the potassium channel of squid axon in a voltage- and time-dependent manner. Onset of block is much more rapid than other symmetric quaternary ammonium ions studied so far (French and Shoukimas, 1981). However, the onset of block is still just detectable under normal solution conditions. Addition of sugar (sucrose or mannitol) to internal and/or external solutions causes a marked slowing of the rate of K-channel block. This finding supports the diffusion limited model for single site block in current use. However, measurements of the temperature dependence of block yield a  $Q_{10}$  much higher than would be expected for a diffusion limited process.

2) The kinetics of both sodium and potassium currents are slowed by the addition of sucrose to perfusion and superfusion solutions. For the sodium currents, the inactivation kinetics are slowed more than activation kinetics. In contrast to changing solvent properties by  $D_2O$  substitution viscosity, changes also reduce the integral of the sodium gating currents, as well as slowing their decay kinetics. Both channels show macroscopic conductance reduction. The data suggest that at least two mechanisms involving water activity are important in channel function.

3) Artificial seawater (ASW) containing 100 millimolar (mM) potassium (K) and 200 to 240 mM cesium (Cs) produces three major effects on squid axon potassium currents. a) These solutions block inward ("tail") currents in a voltage-dependent manner similar to that previously reported from this laboratory for lower concentrations of Cs. (That is, lower concentrations of Cs with respect to external K concentration). b) They partially block outward currents, which suggests that membrane depolarization does not completely relieve blockade contrary to results obtained with lower concentrations of Cs. c) ASW containing 340 Cs or rubidium (Rb) reversibly induces a nonlinear leakage current at potentials positive to 0 mV. This current and the potassium channel current are both blocked by the addition of 300 mM Cs to the internal perfusate. The voltage dependence of the Cs-induced leak current is approximately the same as that of the steady-state current of the Cs-modified potassium channels. These results suggest that 340 mM external Cs or Rb induces a time-independent pathway for outward current flow associated with current through the gated, time-dependent potassium channels. This is a novel finding for biological membranes.

The activation of potassium conductance in squid axons by membrane depolarization is delayed when the depolarizing pulse is preceded by membrane hyperpolarization. The control and the delayed kinetics almost exactly superpose when the control conductance curve is translated along the time axis by a time equal to the hyperpolarizing induced delay (Cole-Moore effect). We have found that the conductance curves do not superpose exactly for all experimental conditions. Specifically, complete superposition fails to occur for depolarizations to +50 and +100 mV. Superposition occurs only for membrane depolarizations in the vicinity of 0 mV. These results are consistent with recent observations from crayfish axons and frog nodes of Ranvier. We have additionally found that the conductance kinetics can be successfully described by incorporating a time dependence into the rate constant for activation of potassium channels in the Hodgkin-Huxley model.

4) The random patterns of single potassium channel openings and closings

preceding and following a voltage clamp step have been obtained using the Hodgkin-Huxley model of potassium channel kinetics. Similar results have been obtained for the Hodgkin-Huxley model action potential.

5) The voltage dependence of the absolute probability for conducting of a stochastically fluctuating, two-state channel requires knowledge of either the single channel conductance in conjunction with the density of channels on the membrane, or an independent measurement of the function of time open of a single channel at one membrane voltage. Gating kinetics for a stochastic single K-channel were derived employing information of the latter type. The time constants,  $\tau_n$ , connecting two steady-state probabilities, are not critically dependent on exact knowledge of an absolute probability. However, the steady-state probability for conducting of a single channel as a function of membrane potential requires, at a minimum, the single channel information listed above. This functional dependence changes qualitatively if the value of the single channel probability for a single voltage is change. The steady-state probability function was determined using the measured  $P(E=-35 \text{ mV})=0.036$ .

#### Publications:

French, R.J. and Shoukimas, J.J.: Blockage of squid axon potassium conductance by internal tetra-n-alkylammonium ions of various sizes. Biophys. J. 34: 271-291, 1981.

Fohlmeister, J.F. and Adelman, W.J., Jr.: Gating kinetics of stochastic single K-channels. In Adelman, W.J., Jr. and Goldman, D.E. (Eds.): The Biophysical Approach to Excitable Systems. New York, Plenum Publishing, 1981, pp. 123-132.

Clay, J.R. and Shlesinger, M.F.: Delayed kinetics of squid axon potassium channels do not always superpose after time translation. Biophys. J. (In press).





Project Description:Objectives.

To examine the structure and possible function of the major filamentous components found in the cytoplasm of neuronal cells: microtubules, neurofilaments, actin filaments and transverse bridges, and to study their ordered association into a transverse bridge lattice system; to investigate the role of this lattice and its possible functional involvement in axoplasmic transport phenomena.

To examine the structure and possible function of neuronal satellite structures, such as the myelin sheath, Schwann and other glial cells.

To develop analytical methods for carrying out the above objective. These include stereoscopic imaging, single displacement optical autocorrelating of electron microscopical images, FFT analysis of line and raster STEM images, and various image filtering and enhancement techniques using the reverse Fourier transform of FFT processed images.

Methods Employed.

Application of Fourier analytical methods to the video line signals comprising the picture raster in scanning transmission electron microscopy (STEM) represents a convenient and objective method for characterization of the periodic structure inherent in many subcellular macromolecular arrays. Among the model systems we have chosen to explore are the network structure in thin sections of embedded tropomyosin crystals, cross sections of myelin sheath and longitudinal sections of squid mantle muscle fibers. In the simplest method, a prominent specimen axis is aligned along or across the line scanning direction in single line mode and the image focused using y-mode presentation. This single line video signal is recorded repetitively (typically 128 or 256 lines) on a digital signal processor to reduce inherent noise and transferred to a computer for analysis. In most instances, the periodicity visible in the full STEM picture can be observed and directly plotted using a cursor on the single line y-mode signal. Examination of the forward Fourier transform of this trace (visualized as a power spectrum) shows a frequency peak corresponding to this spacing together with extraneous lower and higher frequency peaks arising from specimen background and other noise. Often, several orders of the fundamental are seen, depending on the nature of the specimen. Background noise arising from specimen irregularities caused by sectioning or staining inhomogeneities can be eliminated by removing the lower and/or higher frequency components before carrying out a reverse Fourier transformation. This Fourier filtering can be applied to whole image data as well.

Major Findings.

Evidence for an ordered neuroplasmic lattice or network in Loligo and Hermisenda axons using stereoscopic and optical autocorrelative techniques in TEM of relatively thick (0.1-0.5  $\mu\text{m}$ ) sections has been extended in scanning transmission electron microscopy (STEM), and by taking advantage of the low chromatic aberration TEM characteristics of the Philips EM400. The neuroplasmic lattice consists primarily of neurofilaments together with their periodically arrayed side projections (40-45 nm apart) which appear to act as cross-bridges.



The lattice often includes neurotubules and other filamentous elements. This lattice structure spatial continuity appears to account for the gel-like character of axoplasm and its anomalously low optical anisotropy. An essentially identical neuroplasmic lattice structure and cross-bridge spacing is observed in *Bufo* peripheral axons both in internodal regions and in constricted zones associated with nodes of Ranvier and Schmidt-Lanterman clefts. In zones of the latter type, the neurofilament packing density often exceeds  $1000/\mu\text{m}^2$ , a value approaching that ( $1283/\mu\text{m}^2$ ) for hexagonal close packing of elements with a lateral spacing of 30 nm. This closer packing of neurofilaments appears to allow better preservation of lattice order during specimen preparation. Imaging of the neuroplasmic lattice in longitudinal sections by STEM has allowed preliminary characterization in terms of cross-bridge spacing by using Fourier analytical methods as described above. In all cases examined, the neuroplasmic lattice showed an apparent longitudinal spacing of 40-45 nm, in good agreement with TEM stereo and autocorrelative findings. The STEM and TEM results both suggest that the cross-bridges might be related to a larger unit cell by screw axis symmetry.

#### Publications.

Hodge, A.J., Adelman, W.J., Jr., Waltz, R.B. and Tyndale, C.L.: Analysis of periodic structure in model subcellular macromolecular arrays by Fourier processing of single line video signals in scanning transmission electron microscopy. IEEE Trans. Biomed. Eng. (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02273-05 LB								
PERIOD COVERED October 1, 1980 to September 30, 1981										
TITLE OF PROJECT (80 characters or less)  An Investigation of Electro-Mechanical Coupling in Excitable Tissues.										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI:</td> <td style="width: 33%;">J. B. Wells</td> <td style="width: 33%;">Research Physiologist</td> <td style="width: 15%;">LB NINCDS</td> </tr> <tr> <td>Other:</td> <td>D. E. Goldman</td> <td>Guest Worker</td> <td>LB NINCDS</td> </tr> </table>			PI:	J. B. Wells	Research Physiologist	LB NINCDS	Other:	D. E. Goldman	Guest Worker	LB NINCDS
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COOPERATING UNITS (if any)  Marine Biological Laboratory, Woods Hole, MA 02543										
LAB/BRANCH Laboratory of Biophysics, IRP										
SECTION Section on Neural Membranes (located at MBL, Woods Hole, MA 02543)										
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205										
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<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS										
SUMMARY OF WORK (200 words or less - underline keywords)  The major portion of the research effort was concerned with <u>mechanoelectric transduction mechanisms</u> in squid giant axons. An input-output relationship was observed and present studies will further define and quantitate this relationship.										

Project Description:Objectives.

The general objectives of the project are to describe the electrical and mechanical responses of excitable membranes to various applied mechanical manipulations as relates to transduction mechanisms.

Specifically, measurements were made of the minimal applied strain required to produce the biphasic electrical signal commonly generated by an axon during stretch. This could serve as a threshold level for strain-evoked electrical responses. This information, plus additional knowledge of the relationship between applied strain and the resulting primary depolarization, provides a basis for quantitative description of the phenomena.

Another objective was to establish the electrical correlates to the mechanical parameters of stretch, such as rate, amplitude and duration. Finally, an investigation was initiated to study the effect of temperature on the electrical responses to membrane strain.

Methods employed.

The squid giant axon, as observed in vivo, is an excitable membrane system which is not protected by the connective tissue investments which generally encapsulate transducer organs. Although this axon has no known mechanoreceptor function, it does show an electrical response to mechanical stimulation and is a very convenient preparation for the kind of experiments needed. Advantage was taken of these characteristics using the isolated, cleaned axon cylinder. A voice coil displacement transducer was used to apply constant velocity stretches to one end of the axon. The other end was impaled with and tied to an axial indwelling glass capillary electrode which was itself mounted on a piezoelectric force transducer. This configuration permitted simultaneous recording of electrical and mechanical events in the membrane and was adopted in order to optimize the area of membrane subjected to strain. Ion substitution and chemical agents, thought to block specific ionic channels, were used to investigate the electrical responses to stretch.

Major findings.

1) Previous work. Earlier studies have established that an important fundamental mechanical property of the axon is its inability to sustain stress during an applied and maintained strain. The rapid decay of tension in the stretched axon accompanies the decay of the initial depolarization with a nearly identical time course. This suggests that a process operating during the decay is common to both mechanical and electrical events.

Also, previous results obtained during investigation of the electrical properties of the preparation showed: a) Elongation of the axon produced an initial membrane depolarization with rate and amplitude corresponding to rate and amplitude of elongation. This depolarization was not influenced by TTX, a specific sodium channel blocker, or by substitution of choline for sodium in the bathing solution. The initial potential change was called the primary depolarization or primary electrical response. b) A secondary or additional depolarization

of the normal preparation followed the primary response, but with variable amplitude and time course. The secondary response is blocked by TTX or by bathing the axon in choline sea water. c) Repolarization, and subsequent hyperpolarization, completed the electrical response to longitudinal stretch. Thus, the typical response to stretch is a biphasic electrical wave with initial depolarization.

2) Current work. The results of recent experiments employing specific channel toxins and ion substitution suggest, first, the primary depolarization is a graded local response dependent on stretch amplitude, but not the presence of sodium ions or active sodium channels. The secondary depolarization, which does depend on active sodium channels, was evoked by the primary response since a minimal amplitude of primary depolarization was required to elicit a significant secondary response.

Second, the last stage of the biphasic response to stretch, the hyperpolarization phase, was dependent on both amplitude and the maintained duration of stretch. Hyperpolarization of the membrane was also seen in the absence of secondary depolarization or active sodium channels.

Finally, the primary response is increased at low temperatures and experiments are now in progress to characterize the nature of the observed enhancement.

#### Proposed Course of Project:

Two pertinent questions have been raised from the work described above. First, what are the ionic mechanisms underlying the primary response which occurs in the absence of conventional active sodium and potassium channels? The most direct way to answer the question is to measure current flow in the voltage clamped axon during stretch. Preliminary experiments have shown this approach to be valid using the configurations described in the methods above. To this end, the fabrication of necessary electronic apparatus for voltage clamp experiments is the rate limiting step and is in progress. Secondly, what is the mechanism through which the primary response is enhanced at low temperatures where the kinetics of conventional membrane channels are normally slowed? It is planned to continue experiments in progress which are investigating these phenomena.

#### Publications:

Goldman, D.E.: Calculation of the electrogenicity of the sodium pump system of the squid giant axon. In Adelman, W.J., Jr. and Goldman, D.E. (Eds.): The Biophysical Approach to Excitable Systems. New York, Plenum Publishing, 1981, pp. 135-149.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02329-04 LB						
PERIOD COVERED October 1, 1980 to September 30, 1981								
TITLE OF PROJECT (80 characters or less) Mechanical properties of resting and stimulated skeletal and/or locomotor muscles.								
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%;"> <tr> <td style="width: 33%;">PI: J. B. Wells</td> <td style="width: 33%;">Research Physiologist</td> <td style="width: 33%;">LB NINCDS</td> </tr> <tr> <td>Other: M. Schoenberg</td> <td>Research Physiologist</td> <td>LPB NIAMDD</td> </tr> </table>			PI: J. B. Wells	Research Physiologist	LB NINCDS	Other: M. Schoenberg	Research Physiologist	LPB NIAMDD
PI: J. B. Wells	Research Physiologist	LB NINCDS						
Other: M. Schoenberg	Research Physiologist	LPB NIAMDD						
COOPERATING UNITS (if any) Laboratory of Physical Biology, NIAMDD, Bethesda, MD 20205 Marine Biological Laboratory, Woods Hole, MA 02543								
LAB/BRANCH Laboratory of Biophysics, IRP								
SECTION Section on Neural Membranes (located at MBL, Woods Hole, MA 02543)								
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205								
TOTAL MANYEARS: 0.1	PROFESSIONAL: 0.1	OTHER: 0.0						
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS								
SUMMARY OF WORK (200 words or less - underline keywords)  <p>The onset of internal activity, independent of tension development, was determined in skeletal muscle. Variations in muscle stiffness during contractile activity were determined and related to internal contractile processes. The measurement techniques used in these experiments were not shown to be influenced by the high viscosity component associated with resting skeletal muscle.</p> <p>This project is herewith terminated.</p>								



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02151-07 LB
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less) Information Processing in Simple Nervous Systems.		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI.: D.L. Alkon Other: J. Shoukimas J. Acosta-Urquidi Y. Goh A. Kuzirian J. Harrigan I. Lederhendler J. Neary S. Leighton J. Buchanan W. Richards S. Senft	Medical Officer Staff Fellow Visiting Fellow Visiting Fellow Extramural Fellow Mariculturist Behaviorist Biochemist Guest Worker Graduate Student Graduate Student Graduate Student	LB NINCDS LB NINCDS LB NINCDS LB NINCDS LB NINCDS MBL MBL MBL LB NINCDS Northeastern U. Princeton U. Washington U.
COOPERATING UNITS (if any) Marine Biological Laboratory, Woods Hole, MA 02543; Northeastern University; Princeton University; Washington University, St. Louis, MO.		
LAB/BRANCH Laboratory of Biophysics, IRP		
SECTION Section on Neural Systems (located at MBL, Woods Hole, MA 02543)		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 9.0	PROFESSIONAL: 8.5	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The principle objective is to study the mechanisms by which simple <u>neural networks</u> process information with particular emphasis on mechanisms of <u>learning</u> . The nervous system of <u>Hermissenda crassicornis</u> has proven to be a good model for information processing at several levels: <u>sensory transduction</u> by photoreceptors and hair cells, analysis of <u>synaptic circuitry</u> , changes in synaptic circuitry produced by conditioning paradigms administered to intact animals, as well as to isolated nervous systems, membrane properties modified by <u>conditioning</u> , identification of critical developmental stages for the neural networks of interest, as well as stages critical for learning. Techniques employed thus far to pursue these questions include simultaneous intracellular recording from multiple neural elements, paired stimulation of the visual and vestibular pathways using a rotating table, iontophoresis of fluorescent dyes and electron dense materials, automated <u>behavioral monitoring</u> of intact <u>Hermissenda</u> , <u>voltage clamp</u> of identified neural elements. Other methods include <u>mariculture</u> , subcellular fractionation, protein phosphorylation analysis, and uptake of neurotransmitter precursors.		

Project Description:Objectives.

1) To study the mechanisms by which simple neural networks process information with particular emphasis on mechanisms of learning. Information processing at several levels is of interest:

- a. Sensory transduction by photoreceptors and hair cells.
- b. Synaptic interactions between primary sensory receptors.
- c. Synaptic interactions between primary and higher order neural elements.
- d. Intersensory communication: e.g., synaptic interaction between the visual and gravitational sensory pathways.
- e. Changes of synaptic interaction produced by conditioning paradigms administered to the intact animals, as well as to the isolated nervous systems.
- f. Membrane and synaptic properties modified by conditioning.
- g. Identification of critical developmental stages of the neural networks studied, as well as stages critical for learning.
- h. Biochemical mechanisms responsible for long-term neural changes with associative learning.

Methods employed.

1) The nudibranch mollusc Hermissenda crassicornis is the principle experimental preparation. Other marine species, including most recently the lobster, Homarus americanus, are also being screened to provide favorable preparations for specific experimental questions. Intracellular recording from several neural cells simultaneously and voltage clamp with two microelectrodes in the same cell have been the main techniques used thus far. Means for simultaneously stimulating the chemosensory, visual and vestibular pathways while recording intracellular potentials have been developed in our laboratory. Iontophoresis of fluorescent dyes (e.g., Procion Yellow) and electron dense materials (e.g., cobalt) are also being used extensively.

2) Other methods allow biochemical, electron microscopic and developmental approaches to the problems of interest. These include mariculture, subcellular fractionation, protein phosphorylation analysis, uptake of neurotransmitter precursors, etc. Automated behavioral monitoring permits long-term studies of intact Hermissenda during associative learning.

Major Findings.

Past work has focused on seven major areas:

- a. Behavioral conditioning with neural correlates.
- b. Cellular conditioning in isolated nervous systems.
- c. Neural network analysis.
- d. Receptor physiology
- e. Voltage clamp of identified neurons.
- f. Biochemistry of Hermissenda neurons.
- g. Neural development.

For the last few years the major focus of the section has been an integrated multidisciplinary effort to determine a neural and a biochemical basis for an associative learning model with the nudibranch mollusc Hermissenda crassicornis. A number of invertebrate species were considered as potential model systems to analyze cellular mechanisms of behavior and learning. These included Tritonia, Aplysia, Pleurobranchia, Helix, Elysia, and Haminoea. The last two have been cultivated within the laboratory and subjected to preliminary electrophysiologic and histologic investigation. The nudibranch mollusc Hermissenda crassicornis, however, has proven to be a most opportune preparation in satisfying the host of constraints which arose from the questions which were asked. With Hermissenda, it has been possible to define a model of associative learning with the same defining features used for vertebrate associative learning. Movement of Hermissenda toward a light source is markedly reduced after repeated pairing of a light stimulus with rotation. This behavioral change is truly associative (i.e., random light and rotation do not produce the effect), persists for at least several days after training and increases with practice. Stimulus specificity for this behavioral change was indicated by the fact that trained animals did not show changes in responsiveness to food. Because of the relative simplicity of the nervous system, it has been possible to ascertain many of the invariant aspects of the three sensory pathways essential to the associative learning model: the visual, statocyst, and chemosensory pathways.

Changes have been found (within these neural systems of Hermissenda) which occurred only in animals subjected to associative learning paradigms and not to control paradigms. For example, with the first associative training procedure used, it was found that hair cells received less excitatory input from ipsilateral Type A photoreceptors after repeated stimulus pairing but not after control training paradigms. Comparable neural modification could be produced while recording intracellularly. Thus, it was possible to monitor the neural changes as they were progressively produced by the associative training procedure.

Behavioral analyses of the main experimental animal Hermissenda crassicornis have ranged from field observations to comparative studies of laboratory-reared and collected species. Findings of the previous year, for instance, showed that the light response involves a preference for certain levels of intensity, and a biphasic approach/withdrawal process which depends on an individual animal's light history. This behavior is consistent with the predictions from a recent model of phototaxis which assumes that species have preferences for optimum levels of ambient illumination. Field observations, on the other hand, indicate that natural Hermissenda populations undergo diurnal vertical migrations which are determined not only by ambient light and temperature conditions, but also by food availability.

Research efforts during the past year have continued within several scientific disciplines to yield new insights into the cellular physiology of associative learning.

#### Neuronal networks:

1) Intracellular recordings from sensory receptors together with central motor neurons are beginning to define the input-output relations of the visual pathway. Intracellular and extracellular recordings have become possible in

behaving animals. This has permitted assessment of individual neuronal activity as it affects behavior. Many neurons at the input, integrative, and motor output stages have already been characterized. Ultimately, a fairly complete description of the visual pathway and the changes it undergoes during learning should be possible.

2) 2-Deoxyglucose. The completeness of this description of the visual pathway has been increased considerably by quantitative application of the 2-deoxyglucose technique to the Hermisenda visual pathway. Autoradiographic analysis of conventional and electron micrographs has helped determine which neurons show the greatest changes of impulse activity in response to light for control, as well as associatively trained animals.

### Biophysics:

Extensive voltage clamp studies of the soma membrane of isolated Type B photoreceptors have now been conducted. These cells (of which there are three in each eye) were shown to undergo primary changes during associative learning: i.e., changes intrinsic to the soma membrane were observed. Because these cells, via synaptic interactions, affect most, if not all, neurons within the visual pathway, their changes could be responsible for the animals' associative learning behavior. In addition to several light induced conductances ( $\text{Na}^+$ ,  $\text{Ca}^{++}$  and  $\text{K}^+$ ), these voltage clamp studies demonstrated two voltage-dependent outward  $\text{K}^+$  agents: a large, fast, early current and a slow, late current. The large, early outward  $\text{K}^+$  current was found to be greatly reduced in associatively trained, but not control, animals. The kinetics of inactivation of this current were also increased for only the trained animals. This decrease of a specific dark  $\text{K}^+$  current with learning is consistent with a number of previous observations. It explains, for instance, the increased input resistance of Type B cells (after the somata were isolated from their axons and synaptic endings) from trained animals, as well as these cells' enhanced and prolonged depolarizing responses to light.

### Biochemistry:

1) Protein kinase. Intracellular iontophoresis of the catalytic subunit of protein kinase was found to cause similar changes of the isolated Type B soma: increased input resistance and enhanced light responses. This effect is consistent with the previous observation that phosphorylation of a specific phosphoprotein band increases for associatively trained, but not control, animals. Similar phosphorylation changes are now beginning to appear after exposing the isolated Hermisenda nervous systems to paired light and rotation.

2) Methylation and protein synthesis. Investigation of these processes are now in progress. Intact animals are given radioactive precursors whose subsequent incorporation, particularly as a function of learning, is analyzed in individual neurons or small neuronal aggregates.

3) Ionic composition. The intracellular ionic constituents are being determined with the electron probe technique, as well as uptake of radioactively labelled species. These methods should make it possible to determine changes of ionic balance across identified neuronal membranes as a function of learning.



## Behavior and Correlated Electrophysiology:

Numerous refinements of the associative learning model have been made during the past year. Enhanced and prolonged behavioral and correlated electrophysiologic changes resulted from additional light stimuli only when paired (with well-defined temporal relationships) with rotation. The specificity of the associative learning was given further credence when the animals' reduced positive phototaxis became more dramatic for a vertical orientation while their normal negative geotaxis (measured in darkness) remain unchanged following the conditioning procedures. The animals' positive phototaxis itself has now been resolved into component parts including a brisk light-dark border avoidance response and an increased activity, in general, in response to light. A more precise description of that part of the animals' response to light which is modified by conditioning will facilitate understanding of the biophysical basis for the learning behavior.

## Proposed Course of Project:

1) Precise analysis of synaptic interactions between cells within the aforementioned neural networks will be continued with the techniques of intracellular recording and iontophoresis. Particular emphasis will be placed on electron microscopic visualization and reconstruction of cell contacts aided by distribution of hydrogen peroxidase within axons and terminal branches. These studies will not be limited, however, to the networks already discussed. The motor units within the sensory pathways (visual, statocyst, and chemosensory) will be identified. In addition, other more evolved animal forms with potentially analyzable neural networks and behavior will be explored.

2) Anatomic, as well as electrophysiologic, correlates of behavioral and developmental changes will be sought. Using voltage clamp techniques, cellular mechanisms responsible for the learning model will be further analyzed. Particular attention will be given to a study of the potential-dependent currents believed to underlie, at least in part, the observed behavioral changes.

3) Biochemical and pharmacologic analyses of relevant neural systems will continue. We will continue to study subcellular and/or biochemical loci at which primary behaviorally-meaningful changes occur. The Type B photoreceptor will be the initial focus of this work.

We plan to identify the mechanisms leading to the observed changes in protein phosphorylation specific to learning in Hermisenda, i.e., involving changes of cyclase, phosphodiesterase, protein kinase or phosphatase activities. It may also be possible to detect changes in levels of cyclic nucleotides by immunocytochemistry. Initial experiments have utilized isolated nervous systems in order to establish the biochemical detection procedures in our laboratory, but more recent studies involve analysis of phosphorylation in individual Hermisenda neurons.

Other mechanisms of post-translational modification will also be explored. Use of the high resolution, two-dimensional method of O'Farrell is planned to determine if specific proteins are methylated or demethylated during associative training.



We also will study the synthesis and modification of gene products in the Hermissenda nervous system by means of high resolution, two-dimensional electrophoresis. O'Farrell has shown that the 50 or so protein bands visible on conventional one-dimensional gels can be resolved into 1100 proteins by combining isoelectric focusing in the first dimension with DSD-slab gel electrophoresis in the second dimension. A modification in the basic O'Farrell technique provides for the separation of nuclear proteins.

4) Behavioral experiments will be continued to further determine the comparability of the Hermissenda associative learning model to associative learning defined for more evolved species.

We will continue ongoing time-lapse studies of behavior so that motor activity patterns and response to food, cover and conspecifics of wild, laboratory-reared, and experimentally manipulated animals can be described under a variety of illumination conditions. To aid in the analysis of the data generated by this approach, a digitizer interfaced with a computer will be used to record the data directly on tape and then analyze it. The relationship of laboratory to natural habitat behaviors will also be pursued by making additional observations of Hermissenda in the wild.

5) Long-term conditioning effects will be investigated. Neural and behavioral changes which last days and weeks will be compared to those already determined. The interaction of developmental processes with these longer-term changes will also be studied.

6) The generality of cellular principles of learning and development determined for relatively "simple" neural systems and the behavior they control will be examined. Ultimately, mechanisms common to organisms with a wide range of evolutionary diversity and complexity may contribute to understanding the human nervous system and may motivate clinical approaches.

#### Publications:

Akaike, T. and Alkon, D.L.: Sensory convergence on central visual neurons in Hermissenda. J. Neurophysiol. 44(3): 501-513, 1980.

Crow, T.J. and Alkon, D.L.: Associative behavioral modification in Hermissenda: cellular correlates. Science 209: 412-414, 1980.

Alkon, D.L.: Membrane depolarization accumulates during acquisition of an associative behavioral change. Science 210: 1375-1376, 1980.

Farley, J. and Alkon, D.L.: Neural organization predicts stimulus specificity for a retained associative behavioral change. Science 210: 1373-1375, 1980.

Alkon, D.L.: Cellular analysis of a gastropod (Hermissenda crassicornis) model of associative learning. Biol. Bull. 159(3): 505-560, 1980.

Stommel, E.W., Stephens, R.E. and Alkon, D.L.: Motile statocyst cilia transmit rather than directly transduce mechanical stimuli. J. Cell Biol. 87: 652-662, 1980.

Grossman, Y., Schmidt, J.A. and Alkon, D.L.: Calcium-dependent potassium conductance in the photoresponse of a nudibranch mollusk. Comp. Biochem. Physiol. 68A: 487-494, 1981.

Kuzirian, A.M., Alkon, D.L. and Harris, L.G.: An infraciliary network in statocyst hair cells. J. Neurocytol. (In press).

Senft, S.L., Allen, R.D., Crow, T. and Alkon, D.L.: Optical sectioning of HRP-stained molluscan photoreceptors. J. Neurosci. Methods (In press).

Jerussi, T. and Alkon, D.L.: Ocular and extraocular responses of identifiable neurons in the pedal ganglia of Hermisenda crassicornis. J. Neurophysiol. (In press).

Neary, J.T., Crow, T. and Alkon, D.L.: Biochemical correlates of associative learning: a change in protein phosphorylation in the nudibranch mollusc Hermisenda crassicornis. Nature (In press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02088-08 LB																				
PERIOD COVERED October 1, 1980 through September 30, 1981																						
TITLE OF PROJECT (80 characters or less)  Function and Structure of Membrane Ionic Channels: Pharmacology and Ionic Selectivity																						
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																						
<table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">G. Ehrenstein</td> <td style="width: 35%;">Research Physicist</td> <td style="width: 15%;">LB NINCDS</td> </tr> <tr> <td></td> <td>L.M. Huang</td> <td>Staff Fellow</td> <td>LB NINCDS</td> </tr> <tr> <td></td> <td>Nava Moran</td> <td>Visiting Fellow</td> <td>LB NINCDS</td> </tr> <tr> <td></td> <td>H. Robert Guy</td> <td>Staff Fellow</td> <td>LB NINCDS</td> </tr> <tr> <td></td> <td>B. Wong</td> <td>Visiting Fellow</td> <td>LB NINCDS</td> </tr> </table>			PI:	G. Ehrenstein	Research Physicist	LB NINCDS		L.M. Huang	Staff Fellow	LB NINCDS		Nava Moran	Visiting Fellow	LB NINCDS		H. Robert Guy	Staff Fellow	LB NINCDS		B. Wong	Visiting Fellow	LB NINCDS
PI:	G. Ehrenstein	Research Physicist	LB NINCDS																			
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INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																						
TOTAL MANYEARS: <div style="text-align: center;">5.0</div>	PROFESSIONAL: <div style="text-align: center;">4.0</div>	OTHER: <div style="text-align: center;">1.0</div>																				
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS																						
SUMMARY OF WORK (200 words or less - underline keywords)  <p>The long-range purpose of this project is to study the structure and function of <u>membrane ionic channels</u> and their interaction with channel-opening and channel-closing drugs.</p> <p>We determined the properties of <u>batrachotoxin-modified sodium channels</u> in neuroblastoma cells using a <u>suction pipet</u> voltage clamp. Our measurements are generally consistent with previous measurements using radioactive uptake methods.</p>																						

## Project Description

Objectives: To determine the mechanism of action of drugs on membrane ionic channels. Particular emphasis is on drugs that are clinically useful and drugs that are useful in determining channel structure and function.

Methods Employed: For the radioactive-uptake method of measuring membrane current, tissue-cultured cells, such as neuroblastoma, are used. Cells are incubated in media containing channel-opening drugs such as batrachotoxin (BTX) for an hour. The uptake of radioactively-labelled sodium ions ( $^{22}\text{Na}^+$ ) is then measured.

For suction-pipet voltage clamp, a small area of membrane is sucked into a pipet, and then the membrane is broken so that the electrodes in the pipet have access to the cell interior. Voltage clamping is performed with these internal electrodes and with separate external electrodes.

Major Findings: Using a model in which anesthetics can act at two separate sites, we have calculated that anesthetic potency can be maintained with reduced side effects by applying two different local anesthetics simultaneously to nerve cells. In general, this reduction becomes more pronounced when either the fraction of channels blocked or the therapeutic ratio is increased. The reduction can be as large as a factor of two.

BTX-modified sodium channels do not inactivate, even at a time scale of seconds. Their activation properties differ from those of normal sodium channels in that they activate more slowly, and in that their  $g$ - $V$  and  $\tau$ - $V$  curves are displaced about 40 mV in the hyperpolarizing direction.

Methods to predict the structures of membrane proteins from their sequences were developed. These methods include predictions of secondary structures and predictions of how  $\alpha$ -helices can stack together to form a barrier between water and the lipid phase of membranes. These methods were applied to apolipoprotein A1 and portions of the postsynaptic acetylcholine receptor. Structural models of these membrane proteins were constructed.

## Publications:

Huang, L.M. and Ehrenstein, G.: Local anesthetics QX 572 and benzocaine act at separate sites on the batrachotoxin-activated sodium channel. J. Gen. Physiol. 77: 137-153, 1981.

Wong, B.S. Quinidine interactions with Myxicola giant axons. Molecular Pharm. (in press).

Wong, B.S. and Binstock, L.: Brief Communication: Inhibition of potassium conductance with external tetraethylammonium ion in Myxicola giant axons. Biophys. J. 32: 1037-1042, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02091-08 LB
PERIOD COVERED <p style="text-align: center;">October 1, 1980 to September 30, 1981</p>		
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Mathematical Modeling</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <div style="display: flex; justify-content: space-between; align-items: center;"> <span>PI: R. FitzHugh</span> <span>Research Physicist</span> <span>LB NINCDS</span> </div>		
COOPERATING UNITS (if any) <p style="text-align: center;">Marine Biological Laboratory, Woods Hole, Massachusetts</p>		
LAB/BRANCH <p style="text-align: center;">Laboratory of Biophysics, IRP</p>		
SECTION <p style="text-align: center;">Section on Molecular Biophysics</p>		
INSTITUTE AND LOCATION <p style="text-align: center;">NINCDS, NIH, Bethesda, Maryland 20205</p>		
TOTAL MANYEARS: <p style="text-align: center;">1.3</p>	PROFESSIONAL: <p style="text-align: center;">1.0</p>	OTHER: <p style="text-align: center;">0.3</p>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <span><input type="checkbox"/> (a) HUMAN SUBJECTS</span> <span><input type="checkbox"/> (b) HUMAN TISSUES</span> <span><input checked="" type="checkbox"/> (c) NEITHER</span> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <span><input type="checkbox"/> (a1) MINORS</span> <span><input type="checkbox"/> (a2) INTERVIEWS</span> </div>		
SUMMARY OF WORK (200 words or less - underline keywords)  <p>Mathematical models for the following phenomena were studied:</p> <ol style="list-style-type: none"> <li>1) The second and third order (nonlinear) components of <u>membrane admittance</u> as measured by adding a sinusoidal signal to a <u>step voltage clamp</u>.</li> <li>2) Signal detection and analysis of the square wave currents from a <u>single channel</u> opening and closing in a membrane, distorted by noise and low-pass filtering.</li> </ol>		



## Project Description

Experiments by Dr. Adelman and others, in which a sinusoidal component is added to a step voltage clamp, in some cases produce current components with double and triple the frequency of the original sinusoid. Formulas for the second and third order (nonlinear) membrane admittances have been derived for the Hodgkin-Huxley equations. Computations of the amplitudes of the currents have shown good agreement with experiments.

A method for the analysis of noisy experimental records of square waves representing the opening and closing of single channels in a membrane has been developed, using statistical detection theory. A computer program has been written to simulate such signals and to test the detection method by comparison of the reconstructed signal with the original one. The results were satisfactory. The more difficult problem of detection of a noisy signal which has been distorted by a low-pass filter is now being studied.

## Publications

FitzHugh, R.: Nonlinear Sinusoidal Currents in the Hodgkin-Huxley Model. In Adelman, W.J. and Goldman, D.E. (Eds.): The Biophysical Approach to Excitable Systems. New York, Plenum Press, 1981, pp. 25-35 (in press).

Z01 NS 02218-06 LB

## PERIOD COVERED

October 1, 1980 to September 30, 1981

## TITLE OF PROJECT (80 characters or less)

Voltage-Dependent Ionic Conductance in Membranes

## NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT

PI:	D.L. Gilbert	Research Physiologist	LB NINCDS
Other:	R.E. Taylor	Research Physiologist	LB NINCDS
	G. Ehrenstein	Research Physicist	LB NINCDS
	H. Lecar	Research Physicist	LB NINCDS
	C. Morris	Postdoctoral Fellow	LB NINCDS

## COOPERATING UNITS (if any)

R.J. Lipicky, Food and Drug Administration; J. Fernandez, UCLA;  
E. Wenkert, Chairman, Dept. of Chemistry, Rice University, Texas

## LAB/BRANCH

Laboratory of Biophysics

## SECTION

Section on Molecular Biophysics

## INSTITUTE AND LOCATION

NINCDS, NIH, Bethesda, Maryland 20205

## TOTAL MANYEARS:

2.6

## PROFESSIONAL:

2.1

## OTHER:

0.5

## CHECK APPROPRIATE BOX(ES)

☐ (a) HUMAN SUBJECTS☐ (b) HUMAN TISSUES☒ (c) NEITHER☐ (a1) MINORS ☐ (a2) INTERVIEWS

## SUMMARY OF WORK (200 words or less - underline keywords)

One goal of this project is to better understand the mechanisms of the ionic conductance in membranes which are voltage-dependent and excitable. Another goal is to determine how drugs influence these channels. These studies involve the use of the squid giant axon and the giant barnacle muscle fiber. We have continued studies on the mechanism of drug-channel interactions in the squid giant axon membrane. In particular, we have studied yohimbine, phenytoin, and perhexiline, and found that all three exhibit voltage-dependent behavior. We have also studied different types of oscillatory behavior in the barnacle muscle fiber, and developed a mathematical model to explain the behavior. The model is based on experimentally-determined properties of potassium channels and calcium channels.

## Project Description

**Objectives:** To clarify the nature of voltage-dependent channels in electrically excitable membrane, and concomitantly the mechanism by which the channels are modified by drugs.

To study drug-receptor interaction at a molecular level where the effect of the drug-receptor interaction is reflected in the number of channels which are capable of conducting current (i.e., open channels).

To provide biophysical information regarding the mechanism of action of two broad categories of drugs: (1) Drugs that induce repetitive electrical activity (perhaps aptly viewed as channel openers), and (2) drugs that inhibit repetitive electrical activity (perhaps aptly viewed as channel closers).

To explore the adequacy of the Hodgkin-Huxley formulation and voltage-clamp methodology as a framework of reference and tool, respectively, for understanding the mechanism of drug action. To deduce from structure-activity relationship studies, models for drug receptor interaction and eventually to evolve suggestions for the development of new drugs that have greater specificity of effects. To further test a "modified kinetics" model for yohimbine action that we have proposed. To characterize oscillatory behavior in axons and to relate this behavior to the properties of ionic channels. To measure the electrical displacement currents caused by the activation of channel protein subunits in the axon sodium channel.

**Methods Employed:** The primary methods employed in this project are voltage-clamping and current-clamping.

In the time domain gating currents are measured, in the absence of ionic currents, by applying voltage-clamped steps of potential and subtracting the linear component of the capacitative current. The latter is measured at large positive or negative potentials where the gating currents are very small. In the foregoing domain the admittance is measured by applying a pseudo-random binary sequence of pulses using voltage clamp. The membrane voltage and current are recorded separately and later transformed using fast Fourier transform computer programs.

**Major Findings:** Drugs often show use-dependence inhibition on excitable membranes, i.e., the extent of inhibition is highly dependent upon the recent history, or "use", of the sodium channels. Pharmacological agents which exhibit use-dependent inhibition, also inhibit the sodium conductance when there has been no recent "use." The latter effect is known as the tonic effect, in contrast to the use-dependent effect. One drug which we have studied extensively for its use-dependent effects on the squid giant axon is yohimbine. In order to understand the mechanism of these two effects, we have begun a study of the drug's structure-activity relationships. Yohimbine possesses three asymmetric carbon atoms giving rise to the normal, pseudo, allo, and epi configurations.

There are reactive groups on carbon positions 16 and 17. Our studies indicate using nine analogs of yohimbine that both normal and allo configurations are active and indistinguishable. Altering the reactive groups on positions 16 and 17 only had some quantitative effect. Our tentative conclusion is that the basic requirement for the use-dependent and tonic effects of yohimbine may reside in the yohimbine skeleton.

Long depolarizations shift the voltage dependence of gating charge movements towards more negative potentials leaving the total amount of charge movement constant. The results, and comparisons with effects on normal sodium currents, are understandable in terms of simplified, physical models which have been developed by us. The same models predict very well the comparisons made with results in the frequency domain.

The experimental phase of the project on voltage oscillations under current clamp in the barnacle giant muscle fiber is completed. The original impetus for this project was to explain the complex pacemaker-like oscillations which occur in this preparation under constant current stimulation. A theoretical model has been developed, using nonlinear mechanics, which explains most of the important features of the system, such as bistability, transition from plateau action potentials to oscillatory behavior, and secular changes induced by  $\text{Ca}^{++}$  ion accumulation. A paper on this work has been accepted for publication in the *Biophysical Journal*.

#### Publications:

Morris, C.E. and Lecar, H.: Voltage oscillations in the barnacle giant muscle fiber under current clamp. Biophys. J. 34, 1981 (in press).

Gilbert, D.L.: Discussion: What controls atmospheric oxygen? Biosystems 12: 123-125, 1980.

Gilbert, D.L. and Lipicky, R.J.: A Model of Drug-Channel Interaction in Squid Axon Membrane. In Adelman, W.J. and Goldman, D.E. (Eds.): The Biophysical Approach to Excitable Systems. New York, Plenum Press, 1981, pp.

Taylor, R.E., Fernandez, J. and Bezanilla, F.: Squid Axon Membrane Low Frequency Dielectric Properties. In Adelman, W.J. and Goldman, D.E. (Eds.): The Biophysical Approach to Excitable Systems. New York, Plenum Press, 1981, pp. 97-106 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 NS 02219-06 LB																
PERIOD COVERED <p style="text-align: center;">October 1, 1980 to September 30, 1981</p>																		
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Structure and function of the perineurium</p>																		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">R.E. Taylor</td> <td style="width: 35%;">Research Physiologist</td> <td style="width: 15%;">LB NINCDS</td> </tr> <tr> <td>Other:</td> <td>S.I. Rapoport</td> <td>Medical Officer, Researcher</td> <td>LN NIA</td> </tr> <tr> <td></td> <td>N. Shinowara</td> <td>Staff Fellow</td> <td>LN NIA</td> </tr> <tr> <td></td> <td>H. Levitan</td> <td>IPA</td> <td>LN NIA</td> </tr> </table>			PI:	R.E. Taylor	Research Physiologist	LB NINCDS	Other:	S.I. Rapoport	Medical Officer, Researcher	LN NIA		N. Shinowara	Staff Fellow	LN NIA		H. Levitan	IPA	LN NIA
PI:	R.E. Taylor	Research Physiologist	LB NINCDS															
Other:	S.I. Rapoport	Medical Officer, Researcher	LN NIA															
	N. Shinowara	Staff Fellow	LN NIA															
	H. Levitan	IPA	LN NIA															
COOPERATING UNITS (if any) <p style="text-align: center;">Laboratory of Neurosciences, NIA</p>																		
LAB/BRANCH <p style="text-align: center;">Laboratory of Biophysics</p>																		
SECTION <p style="text-align: center;">Section on Molecular Biophysics</p>																		
INSTITUTE AND LOCATION <p style="text-align: center;">NINCDS, NIH, Bethesda, Maryland 20205</p>																		
TOTAL MANYEARS: <p style="text-align: center;">0.4</p>	PROFESSIONAL: <p style="text-align: center;">0.2</p>	OTHER: <p style="text-align: center;">0.2</p>																
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <span><input type="checkbox"/> (a) HUMAN SUBJECTS</span> <span><input type="checkbox"/> (b) HUMAN TISSUES</span> <span><input checked="" type="checkbox"/> (c) NEITHER</span> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <span><input type="checkbox"/> (a1) MINORS</span> <span><input type="checkbox"/> (a2) INTERVIEWS</span> </div>																		
SUMMARY OF WORK (200 words or less - underline keywords)  <p>           We demonstrated the <u>multilayer</u> nature of the <u>perineurium</u> and the role of <u>intercellular tight junctions</u> in maintaining structural and functional integrity. Passing AC current across the perineurium demonstrated that its electrical properties could be represented by two resistances and two capacitances. A high capacitance, which could be ascribed to polarization of charge, probably represents the properties of the intercellular tight junctions.         </p>																		



## Project Description

**Objectives:** To determine how and to what extent the perineurium is involved in the maintenance and regulation of the ionic and metabolic environment of axons and peripheral nerves. The extracellular space in the endoneurium of peripheral nerve is isolated by the endothelial lining of cell capillaries and by the simple layer of cells in the perineurium which are connected together by tight junctions. This project is concerned with the study of the role of the epithelial cell layer in the perineurium.

**Methods Employed:** The methods employed are principally the standard techniques used to study unidirectional fluxes of various substances across the isolated and perfused perineurial sheath of the frog or toad, including the use of radioactive tracers. In addition, histological techniques are employed using electron microscopy, and electrical measurements are made using internal and external voltage and current supplying electrodes.

**Major Findings:** It was demonstrated that the perineurium of the frog sciatic nerve consists of 7 to 19 concentric layers of flattened cells, interspersed by collagen fibers. Close membrane appositions of tight junctions were observed between cells throughout the cell layers. The flat cells of the perineurial layers are characterized by the presence of numerous pits and vesicles at or near the surface membrane.

In order to examine the electrical properties of the perfused perineurium, an AC current was passed across the perineurial cylinder wall and voltage across the wall was measured. Frequency of current varied from 1 Hz to 0.1 MHz. The DC resistance of the perineurium was  $430 \text{ ohm cm}^2$ , and impedance measurements demonstrated two dispersions with center frequencies of 5 KHz and 20 Hz. Exposure to high conductance Ringers decreased the DC resistance. Analysis of the data in terms of a four-element model (two resistors and two capacitances) for an equivalent circuit suggested that small ( $0.1 \text{ } \mu\text{F cm}^2$ ) and large ( $20 \text{ } \mu\text{F.cm}^2$ ) capacitances exist in the perineurium. A small capacitance indicates the presence of 6 or more layers of cells and was unchanged by experimental manipulations. The large capacitance could be ascribed to polarization of charge, perhaps at intercellular tight junctions.

We are continuing work on characterizing the transport and permeability properties of the perineurium, and on the changes in the perineurium as nerves regenerate following Wallerian degeneration.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 NS 02316-04 LB
PERIOD COVERED		
TITLE OF PROJECT (80 characters or less)  Comparison of Different Modes of Axonal Stimulation		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:            G. Ehrenstein	Research Physicist	LB NINCDS
Other:        B. Wong	Visiting Fellow	LB NINCDS
COOPERATING UNITS (if any)  G. Ganot, Technion Medical School, Haifa, Israel		
LAB/BRANCH Laboratory of Biophysics, IRP		
SECTION Section on Molecular Biophysics		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.4	PROFESSIONAL: 0.2	OTHER: 0.2
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
<p>Reversal potentials for two different current components in <u>Myxicola</u> were measured. One component is that induced by <u>mechanical stimulation</u> of the axon and the other component is the <u>leakage current</u>. Both components had reversal potentials of about -45 mV, suggesting that they have a common pathway.</p>		

## Project Description

**Objectives:** To determine the mechanism for mechanical transduction in axons, and to compare this with mechanical transduction in other tissues. To compare the responses of ionic channels to electrical, chemical, and mechanical stimulation.

**Methods Employed:** For the measurement of mechanically-induced membrane current, a giant axon was mounted horizontally in a standard voltage clamp chamber. Mechanical stimuli were supplied to the axon from above by the movement of a loudspeaker driven by a power amplifier and controlled by a pulse generator. The loudspeaker was coupled to the axon by means of a thin metal rod connected to a plastic stylus 2 mm in diameter. This assembly was positioned just above the voltage sensor of the internal electrode of the voltage clamp. Movement of the stylus interrupted the signal between a light-emitting diode (LED) and a photoresistor so that the output of the photoresistor monitored the position of the stylus. The rise time for the stylus movement was about 1 msec.

For the measurement of leakage current, a standard giant axon voltage clamp was employed.

**Major Findings:** The mechanically-stimulated component of membrane current was not affected by tetrodotoxin (TTX), indicating that it does not act through the sodium channels responsible for the action potential.

The mechanically-stimulated component of membrane current has a reversal potential of about -45 mV for small stimulus amplitude, but this increases to about 0 mV for large stimulus amplitudes. The small-stimulus reversal potential is about the same as the leakage reversal potential.

A model to explain these observations is that there are nonspecific pathways normally present in axonal membrane, which are responsible for the leakage current. When a small mechanical stimulus is applied, the current increases through these pathways, but when a large stimulus is applied, the effective diameter of these pathways increases, resulting in a decrease in ionic selectivity.

## Publications:

Ehrenstein, G. and Ganot, G.: Increases in Membrane Conductance caused by Electrical, Chemical and Mechanical Stimuli. In Adelman, W.J. and Goldman, D.E. (Eds.): The Biophysical Approach to Excitable Systems. New York, Plenum Press, 1981, pp. 185-195 (in press).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02317-04-LB
PERIOD COVERED <u>October 1, 1980 to September 30, 1981</u>		
TITLE OF PROJECT (80 characters or less)  Excitable Membranes of Tissue-cultured Nerve and Muscle Cells		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:  Other:	H. Lecar  M. Jackson C. Morris B. Wong G. Ubom	Research Physicist  Staff Fellow Postdoctoral Fellow Postdoctoral Fellow Postdoctoral Fellow
		LB NINCDS  LB NINCDS LB NINCDS LB NINCDS LB NINCDS
COOPERATING UNITS (if any) Medical Neurology Branch, NINCDS; Laboratory of Neurophysiology, NINCDS; Laboratory of Developmental Neurobiology, NICHD; Laboratory of Biochemi- cal Genetics, NHLBI; Dr. A. Mauro, The Rockefeller University, N.Y.		
LAB/BRANCH <u>Laboratory of Biophysics</u>		
SECTION <u>Section on Molecular Biophysics</u>		
INSTITUTE AND LOCATION <u>NINCDS, NIH, Bethesda, Maryland 20205</u>		
TOTAL MANYEARS:  <u>4.8</u>	PROFESSIONAL:  <u>4.0</u>	OTHER:  <u>0.8</u>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  <u>Ionic-current jumps</u> caused by the activation of individual trans- membrane molecular channels are recorded using the external patch elec- trode technique. <u>Unit conductances</u> and <u>gating kinetics</u> are studied in the postsynaptic membranes of cultured rat, human and chick muscle and also in mouse spinal cord neurons. Gating kinetics are determined for various agonists and partial agonists, as cells undergo developmental change or are changed by altered membrane lipid environment. Electron spin reso- nance and <u>fluorescence</u> measurements are done on <u>acetylcholine-receptor pro-</u> <u>tein</u> isolated from electroplox in order to develop a molecular probe for the <u>conformation changes</u> induced by agonists.		

## Project Description:

**Objectives:** To determine the unit conductances and gating dynamics of ionic channels in excitable membranes. To determine the effects of agonists, partial agonists, and antagonists on postsynaptic membranes of nerve and muscle during different stages of development and with altered membrane fatty acid composition. To determine the mechanism of receptor potential generation in stretch receptor neurons. To record the initial appearance of excitability in very young cells. To develop spin-label and fluorescence assays for agonist-induced conformation changes in the nicotinic receptor.

**Methods Employed:** An electrically isolated patch recording system is used to record picoampere-level currents from a  $5 \mu\text{m}^2$  area of cell membrane. Specially fabricated micropipettes when pressed against the cell surface form a bond with the membrane, isolating the microscopic bleb of membrane sucked into the patch. The system is capable of recording the currents caused by activation of individual ionic channels. Trains of single channel current jumps are analyzed automatically by minicomputer, and the results are compared to detailed stochastic models of the gating process. In one series of experiments the patch electrode was used to record ordinary action potentials, excitatory, and inhibitory postsynaptic potentials in cells too small to be penetrated with microelectrodes. Electron paramagnetic resonance and fluorescence spectroscopy is performed on acetylcholine receptor protein isolated from Torpedo electroplax.

**Major Findings:** During the last year single-channel currents were studied in the following systems: cholinergic postsynaptic channels in rat and chick myotubes; inhibitory postsynaptic channels in mouse spinal cord neurons; postsynaptic cholinergic channels in human satellite cells; complement-induced channels in antibody-antigen treated muscle cells. Work is also proceeding on application of the patch technique to the serotonin sensitive neuroblastoma-glioma hybrid and the crayfish stretch receptor neuron.

The search for some way of characterizing the distribution of junctional and extrajunctional channels in rat myotubes led us to other developmental effects involving cholinergic channels. One such effect is the curare-induced depolarization which occurs in embryonic muscle and disappears in later stages of development. We were able to show that this effect has its origin in transient channel openings of the usual conductance value but of very short duration (of the order of 300 microsec). These very short openings from a nominal channel blocker disappear in later stages of development. They also have a voltage-dependent frequency of occurrence which may explain the known voltage dependence of the curare-induced macroscopic depolarization.

The discovery of the short-lived curare channel openings led us to speculate that the distinctions between substances labelled as agonists, antagonists and partial agonists may be a quantitative one. That is, a given agonist has a certain mean occupancy time at a receptor site, during which time the channel flickers open and closed with different transition probabilities, depending on the agonist. Such channel flickering is readily seen with the agonist suberyl-



dicholine. From this point of view, an antagonist is a substance with a long occupancy time and short open lifetimes (perhaps so short as to be unobservable). An effective agonist is a substance that produces flickerings of high duty cycle, and a partial agonist has kinetics which are somewhere in between. Experiments with the partial agonists choline, decamethonium, and succinyl choline so far agree with this conjecture, in that they yield normal-amplitude channels of short duration.

As a method of simulating membrane changes which may occur during development or possibly in myotonic disease states, we are working with cells whose fatty-acid composition has been altered during growth. Preliminary results show that cholinergic channels have altered kinetics (considerably shortened open state lifetimes) when the cell membrane is enriched with oleic acid. Other fatty acids are now being tried. Since these results suggest an indirect interaction between the state of the lipid moiety and the protein receptors, we are also attempting to develop an independent assay for the state of the lipid membrane. At present we are attempting to employ spin-labelled fatty acids and use electron spin resonance spectroscopy as an assay for membrane fluidity to test whether it is the microviscosity of the membrane or some more specialized feature which accounts for the altered gating kinetics of the channels.

Experiments on human acetylcholine receptors have been completed and the results are being submitted for publication. Since the human satellite cells obtained from biopsies resemble embryonic cells, we tested for curare-induced channel activity in these cells. The curare effects were found to be similar to those in rat muscle. In human muscle cells we also studied the interactions between curare and an applied agonist. The observed effects, which may be explained in terms of a channel having two receptor sites, resemble effects that have sometimes been referred to as modulation of the receptor by an applied drug. The major feature of the human muscle studies with three agonists, acetylcholine, carbamylcholine, and suberyldicholine is that there appears to be an additional kinetic component of short channel openings, so that the distribution of channel open times is best fit by the superposition of two first-order processes. Such fast components of channel kinetics are more easily seen in single channel jump experiments than in the more usual noise analysis. We explored the possibility that the two kinetic components might represent two populations of channels analogous to the junctional and extrajunctional channels found in intact innervated muscle. Consequently, we did experiments in which we followed the proportion of the two components as cell development progressed. Young cells had a constant ratio of the two components, whereas older cells showed rather large spreads.

In two separate projects we have extended the study of single channels to postsynaptic channels found in neurons. We have successfully observed single channel currents induced by GABA, muscimol, and pentobarbital in the inhibitory postsynaptic channels of mouse spinal cord neurons. The results confirmed the earlier inferences from noise measurements that these agonists operate on a chloride permeable channel and that the mushroom toxin, muscimol, can hold this channel open longer than the endogenous transmitter, GABA. The other CNS transmission which we are studying is the serotonin response in neuroblastoma-glioma

hybrid cells. Early attempts to observe these channels were unsuccessful and we have since done voltage clamp and pharmacological experiments in order to better characterize this response. One novel feature of the voltage clamp results is the finding of a negative-resistance region in the serotonin-induced current-voltage curve. This effect is similar to the voltage cutoff of the depolarizing curare response and suggests again the possibility of a fairly strong effect of transmembrane potential on the channel gating or agonist-reception kinetics. Noise experiments are planned with a view to determining the serotonin channel conductance and the distribution of channels about the cell surface. Since the serotonin response and its pharmacological perturbation constitute an interesting model for cellular effects relevant to schizophrenia, further efforts will be devoted to this somewhat difficult preparation.

Work has begun on experiments designed to see stretch-induced channel activity in the crayfish stretch receptor. This preparation presents major problems in design of an experimental chamber and in selective enzymatic digestion of surrounding connective tissue. The chamber and muscle-stretcher were fabricated and preliminary enzyme experiments are under way.

The experiments with complement-induced channels in antibody-antigen treated muscle cells are completed and a paper has been accepted for publication. Flickering complement channels with very variable and complex activation kinetics are observed when a complement-containing pipette is pressed against the membrane. Control experiments show that serum in which the complement components are fixed does not produce this effect. Further experiments designed to ascertain whether these flickering complement channels are the actual lytic attack complex or some precursor will proceed using purified complement components.

Preliminary experiments with fluorescence labelled acetylcholine receptor protein demonstrate signal changes in the presence of acetylcholine and suberyldicholine. These changes take time of the order of minutes to reach full strength, and are blocked by antagonists such as bungarotoxin. One possibility which is being explored is that the conformation change coincides with channel desensitization, a conformation change observed in electrophysiological experiments with these compounds and having a similar time course.

Significance to Biomedical Research: The single channel technique allows us to identify and study postsynaptic channels in a way that is particularly simple. In this way, the variety of excitable cell behavior is being reduced to the understanding of the properties of a number of membrane proteins which act as agonist-activated ionic channels. Experimental study of the channels of tissue-cultured nerve and muscle cells allows us to characterize the function of these units by direct means which do not give ambiguous results for complex cells in which several types of channels coexist. The channel kinetic studies not only bear on the mechanism of nerve excitation, but also provide a means for studying the dynamics of conformation change for important membrane proteins in vivo.

The developmental and fatty acid experiments simulate membrane changes which may be of significance for generalized membrane disorders such as myotonia. The

CNS neuron studies provide an approach to neurotransmitter study on a variety of cells which would not be accessible in other ways.

Proposed Course of Project: The patch electrode experiments will be continued. We are developing two innovations which allow recording from an isolated excised patch of membrane and voltage clamp control across the excised patch. With these modifications, the patch electrode method can be extended to a large variety of membrane phenomena which have not yet been accurately characterized. One area of greater emphasis for us will be to apply these methods to basic sensory transduction in mechanoreceptive and chemoreceptive cells. The various molecular probe experiments will be extended as an approach to understanding the structural basis of the gating conformation change during excitation.

Publications:

Lecar, H. and Sachs, F.: Membrane Noise Analysis. In Nelson, P.G. and Lieberman, M. (Eds.): Excitable Cells in Tissue Culture. New York, Plenum Press, 1981, pp. 137-172.

Jackson, M.B. and Berkowitz, S.A.: Nucleation and the kinetics of microtubule assembly. Proc. Natl. Acad. Sci. USA 77: 7302-7305, 1980.

Jackson, M.B., Stephens, C.L. and Lecar, H.: Single channel currents induced by complement in antibody-coated cell membranes. Proc. Natl. Acad. Sci. (in press).

Lecar, H.: Single channel conductance and models of transport. In Adelman, W. J. and Goldman, D.E. (Eds.): The Biophysical Approach to Excitable Systems. New York, Plenum Press, 1981, pp. 109-121 (in press).







# ANNUAL REPORT

October 1, 1980 through September 30, 1981

Laboratory of Neurochemistry  
National Institute of Neurological and Communicative Disorders and Stroke

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ANNUAL REPORT  
October 1, 1980 through September 30, 1981  
Laboratory of Neurochemistry, Intramural Research  
National Institute of Neurological and Communicative  
Disorders and Stroke  
Janet V. Passonneau, Chief

The Laboratory of Neurochemistry is composed of four sections, the Section on Cellular Neurochemistry, the Section on Neurochemical Pharmacology, the Section on Enzymes, and the Section on Neuronal Development and Regeneration. These sections are engaged in a variety of projects.

Section on Cellular Neurochemistry

The Section on Cellular Neurochemistry has four projects currently in progress:

a. Metabolic Profiles in Normal and Diseased Retina.

The study of the energy metabolism of normal retinas is being continued in frogs that are either dark adapted or that have been exposed to 2 seconds or 2 minutes of light. The exposure of photoreceptors to light results in the closing of the Na<sup>+</sup> channels, hyperpolarization and presumably lowered energy utilization. ATP, ATP plus ADP, AMP and P-Creatine are being measured in eight individual layers of the retina to determine the effect of light and exposure time on these energy metabolites.

The effect of starvation followed by refeeding of glucose, glutamine, or both on cultured chick pigment epithelial cells was studied to determine the possible role of glutamine in the energy metabolism of these cells. ATP and P-creatine levels were measured on cell extracts in baseline and experimental cultures.

Granular dystrophy of the cornea is a dominant autosomal linked lesion of the cornea characterized by the accumulation of opaque granular aggregates in the stroma which can eventually lead to impairment of the vision. The metabolic pathway leading to the expression of this disease is unknown. In collaboration with Dr. Merlyn Rodrigues of the Eye Institute, we are studying the biochemical aspects of this metabolic anomaly. By using micro techniques, the protein composition of the corneas of patients with granular dystrophy has been analyzed using corneal biopsies. The result suggests that there is a high molecular weight protein present in the diseased corneas which is not found in normal cornea. In addition, we found an accumulation of higher than normal amount of a second protein in the diseased cornea with electrophoretic mobility similar to that of a keratin found in normal cornea. The use of monoclonal antibodies to verify the nature of this second protein is being carried out with the collaboration of Dr. Tang Tien Sun of the Johns Hopkins University.

## b. Coordinate Effects of Amphetamine on Brain Energy Metabolism and Protein Synthesis.

Amphetamine has been shown to have opposite effects on the body temperatures of rodents, depending on the ambient temperature at which the animals are housed. At temperatures greater than 20° C the drug elevates body temperature, while at ambient temperatures below 15° C body temperatures are lowered, with smaller, less consistent effects in the range between.

Amphetamine inhibits brain protein synthesis by some mechanism which seems to be associated with a hyperthermic response to the drug. Amphetamine also dramatically lowers brain glycogen levels and moderately lowers levels of brain high-energy phosphate compounds. We have now shown that these latter effects of amphetamine on brain energy metabolism in the mouse are also dependent on drug-induced hyperthermia. Thus there is good evidence for a coordinate effect of amphetamine on brain energy metabolism and protein synthesis. There are other experimental treatments which simultaneously alter brain energy metabolism and protein synthesis (e.g., electroconvulsive shock, convulsant drugs, spreading depression, transient ischemia), and a common metabolic response may therefore be responsible for the reduction in protein synthesis under these various conditions.

The nature of the biochemical link between these processes remains to be established. We have found that brain GTP levels are much more affected by amphetamine than are brain ATP levels, suggesting that the availability of GTP may be one factor leading to the reduction of brain protein synthesis by amphetamine.

Future research plans include the measurement of the enzymes regulating glycogen synthesis and breakdown following amphetamine administration. A method will be developed utilizing in vitro incorporation of amino acids by brain extracts, to replace the relatively cumbersome procedure of preparing polysome profiles as a measure of brain protein synthesis. Purine nucleosides and bases will be assigned in brain extracts using high performance liquid chromatography to complement the enzymatic analyses of nucleotide metabolism which have been done so far. Finally, the role of hyperthermia per se in the effects of amphetamine on brain metabolism will be investigated using experimental procedures in which body temperature is elevated by other means.

## c. Characterization of Anaerobic Metabolism of Normal and Neoplastic Astrocytes in vitro

We examined several aspects of the glycolysis of cultured astrocytoma grades III and IV including: glucose uptake, lactate and pyruvate efflux, glutamate and glutamine uptake, and glycogen levels. We concluded the following:

(1) The rates of 18 F-2-deoxy-d-glucose uptake measured in tumors in situ (by PECT scanning) is comparable to the rate of glucose uptake in tissue culture lines derived from tumors at the time of surgery. Differences in the rates of glucose uptake between lines derived from different histological grades of tumor were not observed.

(2) Fifty per cent or more of the glucose consumed appears as medium lactate or pyruvate. This suggests that no more than 50% of consumed glucose is aerobically metabolized. The aerobic glycolytic rate does not correlate with the histological grade of the tumor of origin.

(3) Glutamate and glutamine (which together comprise approximately 1.6 mM in medium) are taken up with a rate comparable to the rate of glucose uptake; this may constitute a significant carbon source for oxidative metabolism.

(4) Each cell line has a characteristic glycogen set-point level. The levels appear to be stable, and not significantly affected by the administration of fresh glucose (refeeding).

(5) We have characterized the cell surface receptor systems in selected astrocytoma grades III and IV, by administering pharmacological agents and scoring for glycogenolysis and/or elevations in cyclic AMP. Each cell line has a characteristic pattern of agents which elevate cyclic AMP or cause glycogenolysis. Most lines show an elevation of cyclic AMP and a glycogenolytic response to catecholamines, and a glycogenolytic response to the calcium ionophore A23187 in the absence of changes in cyclic AMP.

#### d. Metabolic Correlates of Neuronal Transmission in the Hippocampal Slice.

The hippocampal slice preparation is used extensively in electrophysiological, neurochemical and developmental studies. All these investigations have one common event, the ischemic period during which the *in vivo* hippocampus is placed in an *in vitro* environment. Yet, little has been done to define the metabolic perturbations which occur during this period, or to ascertain the recovery period required to reach a new metabolic steady-state within the slice. The goals of this study are two-fold: First, to define the metabolic changes which occur during the initial, *in vivo*, ischemia and the following *in vitro* recovery period; secondly, to compare the metabolic profiles observed above with those of an *in vitro* model of ischemia in which the slices are superfused with a modified bicarbonate buffer devoid of glucose and oxygen. In both instances, we attempt to associate the post-ischemic recovery of the steady-state metabolic profile to the return of electrical activity. Synaptic transmission is evaluated by monitoring the magnitude and shape of the evoked potential in the dentate gyrus. Thus, this study allows correlation of changes in metabolite levels with electrophysiological function in the slice.

Adenylates (ATP, ADP, AMP), phosphocreatine, creatine, cAMP, cGMP, lactate, pyruvate and GABA are measured in slices exposed to a maximum of 15 minutes of ischemia and in slices allowed to recover up to 45 minutes in standard superfusate. This recovery period is sufficient for most of the metabolite levels to re-stabilize. The *in vitro* steady-state levels differ significantly from those observed in the *in vivo* hippocampus of guinea pigs anesthetized with phenobarbital prior to *in situ* fixation. Of interest is the drain on the adenylate and total creatine pools, and the elevated levels of the cyclic nucleotides. Metabolic recovery from 15 minutes of ischemia is essentially the same as that following a 7 minute ischemic episode, though the recovery of the evoked response is delayed and markedly depressed in the 15 minute group.



Energy charge, intracellular pH and NAD/NADH are calculated from the metabolite levels. Following cellular acidification during ischemia, intracellular pH (calculated from the creatine kinase equilibrium) returns to control levels within 10 to 15 minutes of recovery. There is no increase in lactate corresponding to the drop in pH in the in vitro model of ischemia. Energy charge stabilizes at 0.80, a value much below that observed in vivo.

Other studies currently underway measure the long-term (4 to 5 hours) effects of incubation on metabolite levels, as well as changes in high energy phosphate levels in the synaptic region of the dentate gyrus which are associated with failure of synaptic transmission during anoxia.

## Section on Neurochemical Pharmacology

### a. Cerebral Ischemia

A number of pathophysiological events have been demonstrated in the cerebral cortex both during and after an ischemic insult in vivo. Regional brain studies have been performed and indicate that the ischemia-related derangements in the metabolism of cyclic AMP, adenylates and P-creatine are not limited to the cerebral cortex. While the magnitude of the responses did vary from region to region, generally the effects were evident in the 7 areas of the brain examined.

Many of the ischemic and postischemic events which occur in vivo can be duplicated in brain slices. The in vitro approach permits a more thorough examination of the kinetic properties of these processes. For example, the total adenylate pool (ATP + ADP + AMP) decreases biphasically during ischemia; the half-time for the fast component is about 4.5 min. Since the initial restoration of ATP is limited by the size of the adenylate pool, the depressed adenylates could be critical to the recovery of brain function. In addition, brain slices have been useful in describing those factors responsible for the large ischemic-induced changes in cyclic AMP.

A model for transient ischemic accidents has been used to examine the short-term effects of oxygen and glucose deprivation in the rat cerebral cortex. Unlike the massive changes which occur with the gerbil model of global ischemia, the only changes were a 5-fold increase in lactate, a 2-fold increase in cyclic AMP and a 50% reduction in P-creatine. A particular advantage of this approach is that the components of the circulating medium can be easily manipulated.

### b. Experimental Seizures

A kindling model has been developed for the investigation of the biochemical changes which give rise to a seizure focus. Once the amygdala of the rats exhibits a fully "kindled" state, the brains of the rats were frozen in situ and the levels of certain metabolites were measured in the amygdala, septum, hippocampus and cerebral cortex. Preliminary results indicate that neither energy nor GABA metabolism are affected in the experimental animals.

### c. Hypothermia

The glycogen levels in the cerebral cortex and cerebellum of the hamster

are relatively high compared to non-hibernating species including the rat, mouse and gerbil. During arousal from the hibernating state, some glycogenolysis does occur; however, the glycogen concentrations are similar in brains from hibernating, cold-adapted and warm-adapted hamsters. The active and total levels of glycogen synthase and phosphorylase were measured in the hamster cerebral cortex. The phosphorylase a activity increased with increasing body temperature and exhibited a positive correlation with the levels of cyclic AMP, whereas that for glycogen synthase appeared to be independent of both body temperature and cyclic AMP. The relative activities of these 2 enzymes in vivo may serve to explain the high brain levels of glycogen in the hamster.

### Section on Neuronal Development and Regeneration

This section is investigating how neurons exert a trophic action on target tissue and exploring the factors that might lead to the use of nervous tissue grafts to aid in the repair of injured, diseased or congenitally defective nerve tissue.

#### a. Neurotrophic Studies

A study has shown that non-gustatory sensory neurons (i.e., neurons that normally do not innervate tissue of the oral cavity) can, after cross-regeneration into denervated tongue tissue, change ordinary lingual epithelial cells into specialized taste cells. This trophic effect is thought to occur because the neuron delivers a factor to the epithelium which causes taste cell formation. The present finding means that some sensory neurons produce the trophic factor even though they are normally performing another sensory function (e.g., acting as a pain axon) or that lingual tissue can induce an indifferent neuron to begin manufacturing the trophic agent. Further studies are planned to try and resolve these possibilities. In another study motor axons were grown into denervated tongue tissue, but no buds appeared nor were any motor axons observed in the epithelium. The motor axons were plentiful in the connective tissue beneath the epithelium and their failure to enter suggests that some property of sensory nerve is missing in motor axons or else that lingual tissue recognizes what type of axon it will allow to enter its epithelium. Further plans include identifying which type of neuron in sensory ganglia (i.e., the large or small ones) is gustatory, which types of epithelium (e.g., esophageal, skin, etc.) can be converted into taste cells and trying to grow buds in culture.

#### b. Neurological graft studies

The prime interest in these studies is to see whether allografts (i.e., a graft between genetically different members of the same species) of nerve or Schwann cells can be used for neurological repairs. Since an allograft confronts the host with transplantation antigens that can lead to immune rejection, experiments have been undertaken to immunosuppress the host or to alter the antigenicity of the allograft. A variety of immunosuppressive drugs have been given to animals receiving neurological allografts and to date only Cyclosporin A has proved effective. However, further studies are needed to determine the minimal immunosuppressive but non-toxic dose of this drug. Cyclosporin must be given continuously since cessation of therapy leads to allograft rejection. Experiments are also being done to determine whether freezing a nerve allograft alters its antigenicity. This study is important since one investigator claimed that

freezing a nerve allograft resulted in its acceptance rather than its rejection. Indeed, it was further stated that freezing might have induced a state of tolerance to the antigens of the allograft. The idea of reducing allograft antigenicity is appealing and several ways of treating the donor or tissue before grafting are being pursued. It might be that graft modification combined with drug immunosuppression will promote the permanent acceptance of neurological allografts.

I. General program.

The overall objective of the section is to investigate enzymology of particular relevance to neural function. The principal area of study for several years has been the mechanism of active transport for sodium and potassium ions. Related projects are directed at elucidation of possible mechanisms for regulating sodium transport and characterization of analogous systems for calcium ion transport. Each of these is discussed below.

II. Studies on the mechanism and structure of the sodium ion active transport system.

A. Background.

Nerve cells function to receive signals, to transmit signals between points in the nervous system, and to modify these signals in the process of transmitting them to other cells. All of these processes require energy derived from cell metabolism. The basic link between cell metabolism and these various neural processes is the generation of a concentration gradient of sodium ions across the outer cell membrane (plasma membrane). Both the electrical activity and the specific neurochemical transmission of signals are derived from this store of potential energy.

The principal mechanism for sodium ion extrusion from animal cells is driven by the free energy of hydrolysis of ATP. This process is mediated by a protein, Na,K-ATPase, which is an integral component of the outer membrane of virtually all animal cells.

Work from this and other laboratories has established that metabolic energy is transferred to the membrane protein by its direct phosphorylation by ATP. This phosphorylation induces a series of structural changes in the ATPase protein and these changes constitute the process that extrudes sodium ions from the cell in exchange for potassium ions. Previous work from this laboratory has included the initial demonstration of the sodium-dependent phosphorylation of the Na,K-ATPase (Fahn, Albers and Koval, 1963), demonstration of the conformational transformation of the Na,K-ATPase consequent to its phosphorylation (Fahn, Albers and Koval, 1966), demonstration of the low-energy nature of the enzyme acylphosphate complexed with ouabain (Albers, Koval and Siegel, 1972), the simultaneous and independent existence of sodium and potassium ion binding sites on the Na,K-ATPase (Albers, Koval and Swann, 1975). More recently we have engaged in a series of studies of the pre-steady state kinetics of the enzyme phosphorylation reactions (Froehlich et al, 1976, 1979, Hobbs et al, 1980 and in press). These studies have been concerned with confirming more directly the earlier evidence for conformational changes accompanying phosphorylation and ligand binding. Part of this study demonstrated the mechanism of inhibition of the Na,K-ATPase by the potent inhibitor, vanadate.

B. Current studies.

From earlier steady-state measurements, several agents have been characterized as acting on the Na,K-ATPase by stabilizing one of the two major conformers of the enzyme. One of these, oligomycin, acts to inhibit ATP hydrolysis although the enzyme can be reversibly phosphorylated by ATP in its presence. It is



thought to stabilize the E1 conformer. Our recent transient-kinetic studies have confirmed this hypothesis and in addition shown that the binding of oligomycin to the Na,K-ATPase requires the presence of sodium ions.

The reversible phosphorylation of the Na,K-ATPase in the presence of oligomycin is demonstrated by the transient-kinetic technique through the rapid dephosphorylation of the enzyme upon addition of ADP. However the time course of this dephosphorylation is found to be biphasic, and the second, slower phase probably represents a complex of the E1 conformer with ATP. This complex is a hypothetical intermediate for which direct evidence had not previously been obtained and is currently under study.

## II. Studies on regulatory mechanisms for the sodium pump.

### A. Background.

Although the primary function of the sodium pump in neurons is undoubtedly that of generating the ionic gradients which produce the resting cell membrane potential and drive various  $\text{Na}^+$  dependent transport systems for neurotransmitters and nutrients, several hypotheses have been advanced for more specialized functions of the sodium pump. With respect to neural function, an interesting hypothesis arises from the observation that a component of resting membrane potential of many cells has characteristics suggesting a direct hyperpolarization resulting from pump activity. This electrogenic sodium pumping is a natural consequence of the observed stoichiometry of 3  $\text{Na}^+$  ions moved outward to only 2  $\text{K}^+$  moved inward per pump cycle. However it remains to be established whether this hyperpolarization is under the sort of regulatory control that would make it an important factor in such processes as synaptic excitability. Other hypotheses suggest that the stoichiometry of  $\text{Na}^+$  pumping relative to ATP hydrolysis may be under some type of regulatory control, thus producing a pump of varying efficiency. This is primarily hypothesized in relation to theories of thermogenesis, regulation of overall cell metabolic rates, and more recently, a theory of the underlying basis of cell transformation by viruses.

These various theories postulate the existence of ancillary regulatory processes: endogenous regulatory substances, modification by protein phosphokinases, etc.

There are in fact recent reports of the isolation of endogenous factors from brain with ouabain-like inhibitory activity. Papers continue to be published claiming significant modification of Na,K-ATPase activity by neurotransmitters. It now seems well established that there are two variants of Na,K-ATPase in brain tissue, one of which appears to be specific to neurons. Also the presence of a protein phosphokinase acting on a subunit of the Na,K-ATPase has been reported.

### B. Current studies.

We are currently investigating the protein phosphokinase activity of brain and Electrophorus electric organ for ability to phosphorylate endogenous Na,K-ATPase and exogenously added purified Na,K-ATPase at sites other than the catalytic site.

Although numerous other membrane proteins are phosphorylated, as demonstrated



by autoradiography of samples subjected to SDS-PAGE, neither endogenous nor exogenous Na,K-ATPase is phosphorylated by endogenous kinases in electric organ tissue. Accordingly we are examining the possibility that the appropriate kinases may require activation as reported for certain other tissues.

### III. Structural studies of the purified Na,K-ATPase.

#### A. Background.

The Na,K-ATPase consists of two types of subunits, alpha and beta, that are thought to exist in 1:1 stoichiometry. However the complexity of their oligomeric association within cell membranes is unresolved. Both subunits are of high molecular weight and, although some preliminary efforts and determining primary structure are in progress in a few laboratories, the ultimate success of these endeavors is not assured. Because these large protein structures exist in a lipid matrix, there are only a few successful instances of obtaining crystalline membrane proteins. Thus the possibility of obtaining detailed three-dimensional structural information about the sodium pump seems remote at present.

Because of the fundamental importance of this system and the number of basic questions about its function that could be answered by structural information, some alternative approach would be valuable. Complex proteins are considered to have evolved from simpler structures with conservation of structural domains that have analogous functions in different proteins. For example, nucleotide binding sites are known to have common structures in a variety of different dehydrogenases. One might envision approaching the structure of a complex protein by delineating each functional domain as a separate entity.

A difficulty with this approach is that much of the important structure is dependent upon precise folding and apposition of primary chains so that functionally competent structures may not be expected to survive procedures that fragment the protein, although occasional successes have occurred. For example, it has been possible to isolate a functionally competent ATPase fragment of the myosin molecule.

We have previously attempted to isolate a fragment of the Na,K-ATPase that might retain ionophoric activity with respect to sodium ions (Shamoo et al, 1973 and later). The limited success of this attempt led to analogous experimentation with the Ca-ATPase of muscle sarcoplasmic reticulum and in this case a fragment with divalent cation-specific ionophoric activity could be isolated.

#### B. Current studies.

From earlier work with antibodies to the individual subunits of Na,K-ATPase (Jean et al, 1974 and later), we were able to demonstrate an antibody that blocked binding of the specific Na,K-ATPase inhibitor, ouabain, to the enzyme. This observation suggests that antibodies may be useful in defining structural domains of large enzymes. We are now developing monoclonal antibodies to the Na,K-ATPase. Upon developing a series of monoclonal antibodies to different sites on the same protein, we expect to use these as reagents to identify functional domains in the intact enzyme and to identify corresponding fragments in proteolytic digests. Thus these antibodies will be useful both in defining function and in aligning fragments to associate structure and

We are also initiating studies in collaboration with the Section on Cellular Neurochemistry on the in vitro biosynthesis of Na,K-ATPase, using mRNA isolated from Electrophorus electric organ. This project will make use of the monoclonal antibodies for study of the processing of newly synthesized precursors of Na,K-ATPase.

#### IV. Calcium metabolism in electric tissue.

##### A. Background.

Calcium ions are known to be involved in important neural functions, in particular in the release of neurotransmitters. Intracellular levels of calcium are regulated primarily by an ATP-dependent pump analogous to the Na,K-ATPase. Most intracellular functions of calcium are thought to be mediated by a regulatory protein, calmodulin. Electrophorus electric organ is a cholinergically innervated tissue that provides an opportunity to study the mechanism of calcium regulation in excitable tissues. It is known to have a high concentration of calmodulin.

##### B. Current studies.

We have developed a relatively simple method for the large scale purification of calmodulin from electric organ and an improved assay system for measuring calmodulin levels. We have also identified and solubilized a membrane-bound Ca-ATPase that is stimulated by calmodulin. Further characterization and purification of this enzyme is in progress.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 00813-20 LNC																
PERIOD COVERED October 1, 1980 to September 30, 1981																		
TITLE OF PROJECT (80 characters or less)  Enzymological Aspects of Neural Functions																		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																		
<table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">Robert W. Albers</td> <td style="width: 30%;">Head, Sect. on Enzyme Chemistry</td> <td style="width: 20%;">LNC NINCDS</td> </tr> <tr> <td>OTHER:</td> <td>Ann S. Hobbs</td> <td>Staff Fellow</td> <td>LNC NINCDS</td> </tr> <tr> <td></td> <td>Stephen P. Chock</td> <td>Research Assoc.</td> <td>LNC NINCDS</td> </tr> <tr> <td></td> <td>Nagaswami Krishnan</td> <td>Consultant</td> <td>LNC NINCDS</td> </tr> </table>			PI:	Robert W. Albers	Head, Sect. on Enzyme Chemistry	LNC NINCDS	OTHER:	Ann S. Hobbs	Staff Fellow	LNC NINCDS		Stephen P. Chock	Research Assoc.	LNC NINCDS		Nagaswami Krishnan	Consultant	LNC NINCDS
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COOPERATING UNITS (if any)  J. P. Froehlich, NIA																		
LAB/BRANCH Laboratory of Neurochemistry																		
SECTION Section on Enzyme Chemistry																		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																		
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SUMMARY OF WORK (200 words or less - underline keywords)  This project is an investigation into the mechanism and structure of the enzyme, <u>Na,K-ATPase</u> , that catalyses the ATP-dependent extrusion of sodium ions from neurons and other cells. Studies are proceeding along the following lines: (a) measurements of the transient (pre-steady state) kinetics of the <u>phosphorylation and dephosphorylation</u> reactions of the Na,K-ATPase; (b) experiments designed to define the relation of structure to function of different domains of the Na,K-ATPase molecule; (c) steady-state kinetic and ligand-binding studies directed toward elucidation of the <u>mechanism of energy transfer</u> from ATP hydrolysis to the ionophoric process.																		

Project Description:

Objectives: These studies are all designed to obtain a detailed description of the structure of the Na,K-ATPase and of the molecular events that produce active transport of sodium ions.

Methods Employed: A major part of this project involves the use of the rapid quenching technique for the measurement of pre-steady state kinetics of the Na,K-ATPase employing radioactive ATP. The apparatus used permits two-stage addition of reagents so that kinetics of reaction intermediates that are not directly accessible to analysis can be indirectly measured by effects on the pseudo-steady state levels of phosphoenzyme and phosphate release.

Standard techniques of tissue fractionation and enzyme purification are employed along with various forms of gel electrophoresis and radioautography in a study of the possible regulation of Na,K-ATPase by protein phosphokinases.

We are developing a new approach to the study of structure-function relationships in large protein using the Na,K-ATPase as a prototype. The strategy depends upon the development of a series of monoclonal antibodies to different sites along the linear polypeptide chain of the enzyme subunits. These antibodies will be employed as "macro-sequencing" reagents for relating domains of the peptide to each other and to the functioning of the intact enzyme.

A collaborative study is also exploring the use of mRNA isolated from electric organ tissue for in vitro biosynthesis of the Na,K-ATPase or its precursor molecules.

Major Findings: The transient kinetic studies have explored two early stages of the Na,K-ATPase mechanism: (1) the binding of the nucleotide substrate to the enzyme previous to the phosphorylation of the enzyme; and (2) the first (reversible) stage of enzyme phosphorylation. These studies have permitted us to estimate the rate constants for these early steps.

Analysis of the kinetics of ADP-dependent dephosphorylation of the Na,K-ATPase has permitted us to show that a major fraction of the enzyme under steady-state conditions is tightly complexed with ATP. We have used oligomycin to isolate the reversible phosphorylation step and have determined that the binding of this inhibitor is dependent on the prior binding of sodium ions.

The protein phosphokinase studies have demonstrated the presence of membrane-bound protein phosphokinases in electric organ membranes. Extensive examination of various reaction conditions have so far not revealed any evidence of phosphorylation of the beta subunit of the Na,K-ATPase by endogenous phosphokinases, either soluble or membrane bound. It may be that such kinases exist in latent form and further studies are examining this question.



Significance to Biomedical Research and the Institute Program: The Na,K-ATPase is the molecular machine within the cell membrane that extrudes sodium ions from within cells in exchange for extracellular potassium ions. This process generates the principal ionic gradients between cells and their external environment. These gradients are the basis of the electrical potential across cell plasma membranes and thus of the nerve action potentials. The potential energy stored in these gradients is also employed in a variety of other transport mechanisms including the cellular uptake of amino acids and many neurotransmitters.

A detailed knowledge of the molecular events in the operation of this machine is a fundamental part of understanding the functioning of cells and particularly of neurons and other excitable cells. This sodium pump constitutes the major energy requirement of brain. It couples metabolic energy to a large array of functions, many of which are specific to neural tissues. Little is as yet known of the regulatory mechanisms that match the metabolic energy consumed and the potential energy stored by the sodium pump to the various functions dependent upon it.

The studies of structure, mechanism, and regulation outlined here should contribute to this basic understanding.

Proposed Course of Project: The transient kinetic studies will be extended to include comparisons of the soluble and membrane-bound ATPases, particularly in terms of possible differences in their cooperative interactions. Also we expect to carry out direct measurements of bound nucleotide levels under conditions comparable to the transient state measurements.

We intend to extend our study of regulatory protein phosphokinases to brain tissue and in particular to determine if the phosphokinase from brain that acts on the beta subunit can also act on the purified ATPase from other tissues. It has been reported that beta subunit phosphorylation modifies the "efficiency" of sodium ion transport but as yet this has not been defined in terms of the mechanism of the enzyme.

#### Publications:

1. Swann, A. C. and Albers, R. W. Na,K-ATPase of mammalian brain: Differential effects on cation affinities of phosphorylation by ATP and acetylphosphate. Arch. Biochem. Biophys. 203:422-427, 1980.
2. Swann, A. C. and Albers, R. W. Temperature effects on cation affinities of the Na,K-ATPase of mammalian brain. Biochim. Biophys. Acta 644: 36-40, 1981.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 01586-14-LNC
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Trophic Function of Neurons		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <div style="display: flex; justify-content: space-between;"> <div style="width: 40%;">           P.I.: Andrew A. Zalewski         </div> <div style="width: 40%; text-align: center;">           Head, Section Neuronal            Development and Regenera-            tion         </div> <div style="width: 20%; text-align: right;">           LNC NINCDS         </div> </div>		
COOPERATING UNITS (if any)  T. H. Oh, Department of Anatomy, University of Maryland V. Verma, Laboratory of Neuropathology and Neuroanatomical Sciences, NINCDS		
LAB/BRANCH Laboratory of Neurochemistry		
SECTION Neuronal Development and Regeneration		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
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CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS         </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div> <div style="display: flex; justify-content: space-between; margin-top: 5px;"> <div> <input type="checkbox"/> (a1) MINORS         </div> <div> <input type="checkbox"/> (a2) INTERVIEWS         </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords)  <p>           The <u>sensory nerve</u> regulates the development, maintenance and regeneration of <u>taste buds</u>. In order to determine whether this effect was specific to nerves which normally innervate oral tissue, a <u>cross-regeneration</u> study was performed in which sensory axons distributing via the vagus nerve to the chest and abdomen were forced to reinnervate denervated tongue tissue. Taste buds reappeared after <u>reinnervation</u> by the normally non-gustatory vagal axons indicating that the effect of nerve in inducing buds is not confined to oral sensory axons. In another study <u>motor axons</u> of the hypoglossal nerve were grown into denervated tongue tissue but, instead of penetrating into the epithelium and inducing buds, these axons merely meandered in the connective tissue beneath the lingual epithelium. This result might mean that tissue structures or components like basement membrane could regulate which type of axons (sensory or motor) enters its epithelium.         </p>		

Project Description:

Objective: Various tissues require a trophic action of nerve for their development, maintenance and/or regeneration. Neurotrophic relationships exist between nerve and skeletal muscle, nerve and taste buds and nerve and regenerating limbs. In all these instances denervation results in a failure of the target tissue to develop or else the target tissue degenerates and/or disappears. The ability of the nerve to manifest its trophic action is not lost after injury because when the nerve is allowed to regenerate back to its target tissue it can restore health to denervated muscle or else cause new oral epithelial cells to differentiate into specialized sensory taste cells. The purpose of this project is to determine the nature, specificity and plasticity of the trophic interaction between nerve and target tissue. Two studies have been performed on taste buds which attempted to answer these questions: (1) Can a normally non-gustatory sensory nerve induce taste bud regeneration or is this phenomenon restricted only to certain oral sensory nerves; (2) What factor(s) might be responsible for the failure of motor axons to cause bud regeneration.

Methods Employed: Both of the above experiments were performed on rats in which the taste buds of the vallate papilla of the tongue were made to disappear as a result of denervation (i.e., the glossopharyngeal nerves were transected). In one group of rats the cervical branch of the vagus nerve was cross-regenerated into the denervated vallate papilla. This was done in order to determine whether normally non-gustatory vagal sensory axons, which innervate tissue in the chest and abdomen, had the capacity to induce bud reformation. In the second study, the hypoglossal nerve was cross-regenerated into the tongue to find out whether its motor axons actually regenerated into the epithelium of the denervated vallate papilla. The later experiment was designed to ascertain whether the basement membrane regulates which type of axon can enter its epithelium. Three to four months were allowed for the cross-regenerated nerves to interact with the tongue tissue at which time tissue sections of the papilla were prepared for the neurohistological evaluation of nerves and taste buds. Some papillae reinnervated by the hypoglossal nerve were examined by electron microscopy (performed with Dr. Vinod of LNNS, NINCDS, NIH).

Major Findings: The cervical branch of the vagus nerve was able to induce taste bud formation. All ten papillae reinnervated by the vagus nerve bore regenerated buds whereas chronically denervated papillae were devoid of them. Hypoglossal motor axons regenerated into the connective tissue of the vallate papilla, but these axons did not penetrate the epithelium which lacked regenerated taste buds. The regrown hypoglossal axons were plentiful and located in the old glossopharyngeal nerve pathways so that they had the opportunity to reach the epithelium of the papilla.

Significance: The ability of normally non-gustatory sensory axons to induce bud regeneration means that this neurotrophic function is not as

specific or restricted as previously believed. Heretofore, it was believed that a type of sensory axon designated as gustatory existed and that these were located only in certain cranial ganglia. However, the present data indicates that other sensory axons can become gustatory if they are allowed to interact with oral epithelium. Since the sensory axon is thought to release a factor into the epithelium which changes the epithelial cells into taste cells, one wonders if this factor is normally present in non-gustatory sensory axons or whether an antecedent event involves its induction in the axon by the target tissue. This issue can only be resolved if and when the trophic factor is isolated and its sources located by immunocytochemical techniques. The failure of hypoglossal axons to penetrate lingual epithelium might explain why the motor axons do not induce bud regeneration, but it does not preclude their capacity to manufacture the trophic factor for taste buds. It might be that some property of the axon such as the presence of an enzyme at the nerve tip might be required to allow the axon to enter the epithelium and deliver the trophic factor. Indeed, all neurons might be trophic but their expression of this property might be limited by unsuspected events occurring at the nerve tip.

#### Proposed Course of Project:

- 1) An attempt will be made to identify the sensory neuron responsible for supporting taste buds. There are two types of neurons in sensory ganglia one of which in the newborn is susceptible to the neurotoxin capsaicin. Capsaicin will be given to newborn rats and if the correct neuron is killed no taste buds should appear in the papillae of the tongue. If this result occurs it will be interesting to determine whether the remaining sensory neurons might, after their axons are transected and allowed to regenerate, become trophic for taste buds.
- 2) An attempt will be made to determine whether epithelium derived from skin, esophagus, cornea, etc. can give rise to taste buds after reinnervation by a gustatory nerve. If these epithelia are unsuccessful they will then be combined with oral dermis (e.g., after initial trypsinization of skin and tongue) to see what, if any, role the dermis plays in imparting specialization to the epithelium it harbors.
- 3) An attempt will be made to grow taste buds in culture. We have tried this but have not been successful even though the neurons and tongue tissue have survived in vitro.

#### Publications:

Zalewski, A. A. Regeneration of taste buds after reinnervation of a denervated tongue papilla by a normally non-gustatory nerve. J. Comp. Neurol. in press (1981).

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> <b>INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER <div style="text-align: center; font-weight: bold;">Z01 NS 02006-09 LNC</div>															
PERIOD COVERED <div style="text-align: center; font-weight: bold;">October 1, 1980 to September 30, 1981</div>																	
TITLE OF PROJECT (80 characters or less) <div style="text-align: center; font-weight: bold;">Regulation of Metabolism in Glioma and Neuroblastoma Cell Lines</div>																	
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																	
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI:</td> <td style="width: 33%;">Janet V. Passonneau</td> <td style="width: 33%;">Chief</td> <td style="width: 10%; text-align: right;">LNC</td> <td style="width: 10%; text-align: right;">NINCDS</td> </tr> <tr> <td>Other:</td> <td>Craig J. Cummins</td> <td>Staff Fellow</td> <td style="text-align: right;">LNC</td> <td style="text-align: right;">NINCDS</td> </tr> <tr> <td></td> <td>Wesley D. Lust</td> <td>Head, Sec. Neurochem. Pharm.</td> <td style="text-align: right;">LNC</td> <td style="text-align: right;">NINCDS</td> </tr> </table>			PI:	Janet V. Passonneau	Chief	LNC	NINCDS	Other:	Craig J. Cummins	Staff Fellow	LNC	NINCDS		Wesley D. Lust	Head, Sec. Neurochem. Pharm.	LNC	NINCDS
PI:	Janet V. Passonneau	Chief	LNC	NINCDS													
Other:	Craig J. Cummins	Staff Fellow	LNC	NINCDS													
	Wesley D. Lust	Head, Sec. Neurochem. Pharm.	LNC	NINCDS													
COOPERATING UNITS (if any) <div style="text-align: center; font-weight: bold;">None</div>																	
LAB/BRANCH <div style="text-align: center; font-weight: bold;">Laboratory of Neurochemistry</div>																	
SECTION <div style="text-align: center; font-weight: bold;">Section on Cellular Neurochemistry</div>																	
INSTITUTE AND LOCATION <div style="text-align: center; font-weight: bold;">NINCDS, NIH, Bethesda, Maryland 20205</div>																	
TOTAL MANYEARS: <div style="text-align: center; font-weight: bold;">1.4</div>	PROFESSIONAL: <div style="text-align: center; font-weight: bold;">1.4</div>	OTHER: <div style="text-align: center; font-weight: bold;">0</div>															
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS   <input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS         </div> <div> <input checked="" type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input type="checkbox"/> (c) NEITHER         </div> </div>																	
SUMMARY OF WORK (200 words or less - underline keywords) <div style="padding: 10px;"> <p>Studies are being carried out on <u>primary cultures</u> of rat brain astrocytes which have been <u>transformed with Herpesvirus</u>, and on cell lines derived from <u>human astrocytomas</u>, types II, III, and IV. The levels of key metabolites, glucose, glycogen, lactate, pyruvate, ATP, P-creatine, GABA, glutamate, glutamine and cyclic nucleotides were measured various times after refeeding confluent cultures with Eagle's Minimal Essential Medium containing glucose. <u>Glycogenolysis</u> in the rodent cell line is regulated by the availability of glucose, or by catecholamine induced increases in cyclic AMP. Alterations in the energy status also are correlated with glycogenolysis. The rate of anaerobic and aerobic glycolysis was examined in the human cell lines. Over 50% of the total glucose consumed appears in the medium as lactate and pyruvate. The <u>amino acids</u> glutamate and glutamine are rapidly taken up and may constitute a quantitatively significant oxidizable carbon source. <u>Glycogen levels</u> are not affected by glucose administration (refeeding), and three of four lines tested showed a catecholamine-induced glycogenolysis.</p> </div>																	



Project Description:

Objectives: To investigate the regulation of glucose and glycogen metabolism in primary, transformed and neoplastic astrocytes in culture.

Methods Employed: The cells are grown in plastic dishes using modified Eagle's Medium containing 10% fetal or newborn calf serum, in a humidified atmosphere of 95% air, and 5% carbon dioxide at 37 degrees centigrade. Extracts of cells are analyzed for metabolites, as well as for cyclic nucleotides and pyridine nucleotides, and for enzymes such as glycogen synthase and glycogen phosphorylase. All of the metabolites have been applied to measurements in whole brain, and are thus easily adapted for use with cells in culture.

Major Findings: We have examined several aspects of glucose metabolism in cultured lines derived from astrocytoma grades II-IV. We were able to use several cell lines derived from tumors, the glucose uptake of which had been determined by 18F-2-deoxy-d-glucose positron emission computed tomography (PECT). The rates of glucose uptake in the cultured tumor lines varied from 0.9 to 3.0 nmoles/min/mg protein which were close to the values of 2-deoxy-d-glucose uptake measured in the tumors in situ by PECT scanning.

In two lines which were examined in detail, over half of glucose taken up in vitro is apparently metabolized only as far as lactate or pyruvate. The rate of glutamine uptake approaches the rate of glucose uptake, and glutamate is taken up as well. Both of these amino acids are taken up with a half time of 6 hrs or less, and if they are completely oxidized, may constitute a quantitatively significant carbon source.

The resting levels (i.e., levels in cultures fed 48 hrs previously) of glycogen are higher than that found in the animal cell line, and there are significant qualitative and quantitative differences between the various human cell lines. All human cell lines show a high resting level of glycogen (from 200-1500 nmoles/mg protein), which does not change after glucose load (refeeding).

Glycogenolysis was extensively studied in both the human and animal cell lines. In the Herpesvirus transformant, glycogenolysis follows the partial depletion of medium glucose, and also proceeds rapidly after beta-adrenergic stimulated increases in cyclic AMP levels. Glycogenolysis follows treatment with anaerobic and aerobic metabolic inhibitors, only if glucose is limiting.

Major differences in glycogenolytic metabolism are found when the human cell lines are compared. These differences are not apparently related to either tumor grade, or time in culture. Four of five cell lines showed a typical beta-adrenergic induced glycogenolysis. The non-responding line showed a two-fold increase in cyclic AMP after administration of isoproterenol, and the one responding cell line tested showed a four fold increase in cyclic AMP. Another cell line showed a glycogenolytic response



after treatment with the alpha-adrenergic agonist phenylephrine, and three of four lines exhibited glycogenolysis after treatment with the calcium ionophore A23187. None of the lines showed a glycogenolytic response to histamine, serotonin, 2-chloro-adenosine or to acetylcholine.

Significance to Biomedical Research and the Program of the Institute:

A study of the regulation of metabolism in the brain is complicated by the presence of several cell types and the inability to determine the site of particular reactions. Such studies are facilitated by the use of primary as well as transformed cell lines in vitro. Although cell lines in culture may substantially differ from their counterparts in situ, they offer a first approach to the problems of regulation of metabolism of brain, which may be pursued in dissociated brain cells.

Since glial tumors in man are a major oncotype of brain tumors, an understanding of the metabolism of transformed glia may elucidate important mechanisms relevant to etiology and treatment. Comparisons of primary and transformed and primary astrocytes, and the experimental transformation of primary cultures may yield important information on the metabolic condition for, and consequences of the transformation process.

Several classes of glycogen-deficient diseases have been well characterized in man, and an increasing number have been described in animals. Collection and characterization of cell lines mutated in specific loci of glycogen metabolism may be useful in the understanding of the biochemical genetics, underlying mechanisms, and possible treatment of individuals affected with glycogen-deficient diseases.

Proposed Course of the Project. Dr. Cummins has taken a position with the Surgical Neurology Branch, NINCDS. The project in this laboratory will thus be discontinued, and further research on human astrocytomas carried out in that Branch.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02142-07 LNC
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less) Cerebral Metabolism in Altered Metabolic States of the CNS		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:  Other:	W. D. Lust J. V. Passonneau H. Arai A. B. Wheaton G. K. Feussner	Head, Sect. Neurochem. Pharm. Head, Sect. Cell. Neurochem. Visiting Fellow Biol. Lab. Tech. (Micro) Chemist     LNC NINCDS LNC NINCDS LNC NINCDS LNC NINCDS LNC NINCDS
COOPERATING UNITS (if any) Department of Neurology, Johns Hopkins School of Medicine, Baltimore, MD		
LAB/BRANCH Laboratory of Neurochemistry		
SECTION Section on Neurochemical Pharmacology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD 20205		
TOTAL MANYEARS: 2.8	PROFESSIONAL: 1.3	OTHER: 1.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Cerebral metabolism in vivo was examined in a variety of neurological states including a) <u>ischemia</u> , b) <u>hypothermia</u> and c) <u>paralysis</u> . a) Previous investigations have centered on the biochemical changes which occur both during and after an ischemic insult. With increasing periods of ischemia, there are major perturbations in the recovery of total adenylates, the cyclic nucleotides, glycogen and P-creatine which may adversely affect the restoration of function. Regional studies clearly indicate that many of these pathophysiological events are not not limited to the cerebral cortex, but occur elsewhere with varying intensity. Brain slice studies indicate that only adenosine, norepinephrine and histamine are sufficiently active to mediate the postischemic rise in cyclic AMP. b) The relative activities of glycogen phosphorylase and synthase were measured as a function of body temperature in brains from hibernating hamsters. The % phosphorylase a increased with increasing body temperature and was directly proportional to the cyclic AMP levels in the brain. c) Experimentally-induced paralysis affecting the spinal cord neurons had little or no effect on cortical metabolites, but significantly decreased cyclic GMP and increased both GABA and glycogen in the cerebellum.		

Project Description:

Objectives: To examine the mechanisms involved in the biochemical perturbations of the brain induced by ischemia and to determine if these events are related to the loss of functional recovery after prolonged periods of ischemia. To investigate the possibility that selective vulnerability of certain neurons to ischemia is a biochemical phenomenon. To compare the biochemical profiles in the hibernating and ischemic brains, both conditions exhibiting an electrically quiescent state of the CNS. To evaluate the effects of spinal paralysis on brain metabolism.

Methods employed: For all the studies, the cyclic nucleotides were measured by immunoassay and the remaining metabolites either by enzymatic analysis or by luciferin-luciferase. In the studies in vivo, the animals were frozen intact in liquid nitrogen or the brains were frozen in situ, a modification of the funnel-freezing technique first described by Kerr (1935). The advantage of the latter procedure, a patent circulation with oxygenation during fixation, permits the sampling of deeper regions of the brain.

a) Ischemia. (in vivo) Mongolian gerbils were anesthetized, the common carotid arteries were exposed and looped with sutures. One day later, the arteries were ligated for the ischemia studies or occluded with aneurysm clips for the recovery stage. In the latter group, the clips were removed at the appropriate times in order to study the recovery process. The brains were removed at  $-20^{\circ}$  and then sectioned at 20 microns. Following vacuum drying at  $-45^{\circ}$ , the discrete areas of the brain to be examined were free-hand dissected and weighed on a quartz fiber balance.

Brain slices: Rats or gerbils were decapitated ('0' time of ischemia), the entire forebrain was removed at  $37^{\circ}$  and the cerebral cortex was sliced and either fixed with perchloric acid (ischemia) or incubated in either oxygenated phosphate-buffered saline containing glucose or oxygenated Krebs bicarbonate buffer containing glucose (recovery).

b) Hypothermia. Hamsters were housed in a  $4^{\circ}$  cold room for approximately 4 months. During the later stages of this period, about 40% of the hamsters hibernated. The animals were either frozen during hibernation (H) or were aroused to a given body temperature and then frozen. Control animals were either warm-adapted (WA) at a normal ambient temperature or were the animals that did not hibernate (CA) at  $4^{\circ}$ .

c) Paralysis. Mice were infected with an AKR virus. Control animals were those not exhibiting any neurological signs of paralysis.

Major Findings: a) The gerbil model of ischemia has been used to investigate the fate of certain energy metabolites, the cyclic nucleotides and some putative neurotransmitters during ischemia and re-circulation. There are generally two types of response: 1) the rapid changes which are generally manifested in the energy metabolites and 2) the slower changes which include a rise in GABA and a fall in cyclic GMP and the total adenylates. While the metabolite response during ischemia reflects the quiescence of the brain, generally the severity of the insult is not indicated.



The recovery phase following an ischemic insult represents a process that is substantially more complex than a mere reversal of the biochemical events which occurred during ischemia. During recirculation, there is a large secondary rise in cyclic AMP, an overshoot of glycogen, glucose and P-creatine and a relatively slow restoration of the adenylate pool (ATP + ADP + AMP). The expression of these events are generally more dramatic after longer periods of ischemia and provide some evidence that certain metabolic abnormalities persist when other parameters indicate a normalization of brain metabolism. In addition, the rate of metabolite restoration is generally slower after longer periods of ischemia. Thus, it is quite possible that the recirculation period has its own set of pathogenic events which arise from the sudden availability of oxygen and glucose to a metabolically and functionally inactive tissue.

Due to the problem of brain fixation, previous studies have focussed on ischemic changes in the cerebral cortex. By using in situ fixation, the effects of ischemia could be examined in various regions of the brain. The results indicate that the changes in cyclic nucleotides, ATP and P-creatine were evident in all six regions of the brain sampled, but to varying degrees. For example, the post-ischemic rise in cyclic AMP was substantially greater in the hippocampus and septum than in the other areas examined. In addition, based on the histological observations that H1 neurons of the hippocampus are more susceptible to ischemia than the H3 neurons, the metabolites were measured in discrete regions of the hippocampus enriched in these particular neurons. In this study, there was little or no biochemical evidence to support the greater vulnerability of the H1 neurons.

Brain Slices. Many of the biochemical events that occur both during and after an ischemic episode in vivo can be mimicked in vitro. As would be expected, the metabolite changes in brain slices in the absence of oxygen and glucose were similar to those observed in vivo. Upon adding glucose and oxygen back to the slice medium, the levels of ATP and P-creatine were regenerated but only to about 50% of the in vivo values. The total adenylates decreased biphasically during ischemia; the initial half-time for the disappearance was 5 min. In contrast to the ATP and P-creatine levels, the total adenylates remained depressed during the recovery period. The postischemic rise in cyclic nucleotides was also evident in the brain slice preparation. The cyclic AMP rise during recovery 1) may increase up to as much as 50-fold, 2) is of limited duration and 3) appears to be greater after longer periods of ischemia. The nature of the response suggests that it may be critical to the restoration of function.

To evaluate the factors which regulate the cyclic AMP levels both during and after ischemia, various adenylate cyclase agonists were examined in brain slices from gerbils. Adenosine, norepinephrine and isoproterenol were sufficiently potent to account for the magnitude of the cyclic AMP accumulation induced by ischemia. There were only marginal effects with histamine and prostaglandin E2, and no detectible changes with serotonin, acetylcholine and dopamine.

b) Hypothermia. The levels of glycogen are substantially higher

in brains from hamsters than those from rats, mice and gerbils. While the concentrations of glycogen in cold-adapted, warm-adapted and hibernating hamsters were not significantly different, the levels did decrease during the arousal period. The active forms of the biosynthetic and degradative enzymes for glycogen were measured as a possible explanation for the above observations. While the % glycogen synthase a increased from 41 to 58% during arousal (body temperatures ranging from 4 to 31°C), the % phosphorylase a increased from 8 in the hibernating brain to 36 at a body temperature of 31°C. There was a direct relationship in the neocortex between the % phosphorylase a and the cyclic AMP levels.

In addition, the NAD/NADH ratio calculated from the lactic dehydrogenase equilibrium was significantly lower in the hibernating brain and the intracellular pH derived from the creatine kinase equilibrium was significantly higher in the hibernating brains.

c) Paralysis. The effect of paralysis (secondary to the virally-induced loss of spinal cord neurons) on the metabolite profile was examined in the cerebellum and cerebral cortex. There were significant differences in metabolites between the group exhibiting neurological signs of paralysis and those that did not. However, the paralyzed mice had a tendency to lose body weight which could, via fixation artefact, account for some of the observed differences. The 50% decrease in cyclic GMP, the 30 and 23 % increase in GABA and glycogen, respectively, in the cerebellum of paralyzed mice were the only effects apparently unrelated to the fixation problem.

#### Significance to Biomedical Research and the Institute Program.

The gerbil model of ischemia in vivo has been particularly useful in defining the abnormal biochemical events which occur both during and after an ischemic event. If brain damage is related to a failure in the biochemical machinery, then further examination of these biochemical perturbations may provide some insight into the pathophysiological mechanisms that lead to brain damage. For example, the large post-ischemic rise in cyclic AMP may be critical to the restoration of brain function. It has been established by a number of investigators that cyclic AMP is inhibitory to the firing rates of certain neurons. It is quite possible that the elevated cyclic AMP could be a determining factor in the onset of electrical activity of the brain during this critical period. Until the cyclic AMP response can be manipulated by an appropriate cyclase blocker, the significance of cyclic AMP to the restoration of function, or the lack of it, really cannot be ascertained. Although many of the other metabolic abnormalities are not directly related to the excitability of the CNS, their impact on the recovery process could be just as great. Normal levels of ATP cannot be regenerated as long as the concentrations of the total adenylates remain depressed. While the adenylates decrease slowly during ischemia, the recovery is also gradual and ATP can only be regenerated to about 90% of the total adenylates. Perhaps, increasing the adenylate pool size would minimize the ischemic brain damage.

The hibernating hamster is a good model for studying the transition of the brain from an active to an inactive state. A major



difference in the cerebral metabolites between an ischemic and a hibernating brain, both in an electrically quiescent state, is that the energy state of the brain from hibernating hamsters is maintained. An elevation of GABA and a decrease in cyclic GMP occurs in both types of brains. Another factor is the large change in cyclic AMP in the ischemic brain which does not occur in the hibernating brain. The differences between these two quiescent brains may be useful in identifying those factors that lead to irreversible brain damage.

Proposed Course of Project: Previous experiments have emphasized the changes in metabolites that occurred in the cerebral cortex. By using sensitive quantitative histochemical methods, the biochemical response to ischemia will be determined in other discrete regions and eventually in single cells. This approach should provide some information relevant to the selective vulnerability of certain populations of cells to ischemia.

Since many of the pathophysiological events which occur in vivo can be reproduced in brain slices, this model can be used to examine the underlying mechanisms. In addition, brain slices may be useful for testing agents that might improve metabolic recovery.

Undoubtedly, many of the cellular responses to ischemia are invoked to preserve the viability of the tissue. It is the other processes that are incompatible with recovery that require attention. Modification of those events may be the first step to improving surviving following an ischemic episode.

#### Publications:

Conger, K.A., Garcia, J.H., Kauffman, F.C., Lust, W.D., Murakami, N. and Passonneau, J.V.: Alanine to glutamate ratios as an index of reversibility of cerebral ischemia in gerbils. Ann. Neurol. 71: 370-382, 1981

Lust, W.D., Murakami, N., de Azeredo, F. and Passonneau, J.V.: A comparison of methods for brain fixation. In: (Passonneau, J.V., Hawkins, R., Lust, W.D. and Welsh, F., eds.) Cerebral Metabolism and Neural Function. Williams and Wilkins, Baltimore, 1980, pp. 10-19.

Murakami, N., Lust, W.D., de Azeredo, F., and Passonneau, J.V.: Ischemic related changes in adenine nucleotide metabolism. In: (Mrsulja, B.B., Rakic, Lj.M., Spatz, M. and Lust, W.D., eds.) The Pathophysiology of Cerebral Metabolism. Plenum Press, New York, 1980, pp. 5-22.

Lust, W.D., Feussner, G.K., Barbehenn, E.K. and Passonneau, J.V.: The enzymatic measurement of adenine nucleotides and P-creatine in picomole amounts. Anal. Biochem. 110: 258-266, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02254-05-LNC
PERIOD COVERED <div style="text-align: center;">October 1, 1980 through September 30, 1981</div>		
TITLE OF PROJECT (80 characters or less) <div style="text-align: center;">The Use of Neurological Grafts to Repair the Injured Peripheral or Central Nervous System</div>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	A. A. Zalewski	LNC      NINCDS
Other:	A. K. Gulati	LNC      NINCDS
COOPERATING UNITS (if any)  <div style="text-align: center;">W. K. Silvers, Department of Human Genetics, University of Pennsylvania</div>		
LAB/BRANCH <div style="text-align: center;">Laboratory of Neurochemistry</div>		
SECTION <div style="text-align: center;">Neuronal Development and Regeneration</div>		
INSTITUTE AND LOCATION <div style="text-align: center;">NINCDS, NIH, Bethesda, Maryland 20205</div>		
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
2.5	1.7	0.8
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div style="width: 30%;"> <input type="checkbox"/> (a) HUMAN SUBJECTS         </div> <div style="width: 30%;"> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div style="width: 30%;"> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div> <div style="display: flex; justify-content: space-between; align-items: flex-start; margin-top: 5px;"> <div style="width: 30%;"> <input type="checkbox"/> (a1) MINDRS         </div> <div style="width: 30%;"> <input type="checkbox"/> (a2) INTERVIEWS         </div> </div>		
SUMMARY OF WORK (200 words or less - underline keywords)		
<p>Host <u>axons</u> can regenerate through a long nerve allograft if the host is made immunologically tolerant to the transplantation antigens of the graft donor. However, if tolerance is abolished allograft rejection occurs and the host axons in the graft degenerate. Since it was also observed that host axons began a second regenerative effort into the remnants of the rejected nerve a study of the outcome of this regrowth was made. Unfortunately, these axons only grew a short distance into the rejected nerve and then they stopped. This result indicates that an inert connective tissue matrix of nerve will not support the growth of axons over any great distance and that viable Schwann cells are needed. The new immunosuppressive drug <u>Cyclosporin A</u> was found to prevent the rejection of a nerve allograft but when therapy was terminated rejection occurred. This finding means that Cyclosporin A has to be given continually and that short-term treatment does not induce tolerance as others have reported. In another study no evidence was found that freezing a nerve allograft altered its antigenicity. Consequently, no host axons grew through frozen nerve allografts and host animals became sensitized to graft antigens.</p>		

Project Description:

Objective: It is now apparent that transplanted nerve and Schwann cells can survive and promote the repair of nervous tissue. For example, sensory nerve cells of the peripheral nervous system can survive transplantation, regenerate their axons and induce the formation of taste buds. In addition, it has been shown the vasopressin neurons in grafts of fetal central nervous system can produce sufficient hormone to partially reverse the polyuria and polydipsia in a rat with congenital diabetes insipidus. Finally, gaps in injured peripheral nerve have been repaired with nerve grafts in which the Schwann cells aid the regeneration of host axons. The major barrier to using nervous tissue allografts (i.e., grafts between genetically different members of the same species) is the presence of transplantation antigens on the grafted cells which evoke an immune response by the host that culminates in allograft rejection. We have begun a series of studies to elucidate the factors which contribute to allograft antigenicity in hope that we might find ways of preventing rejection. In one study with nerve allografts, rats were made tolerant to donor antigens so that allograft rejection would not occur. As expected, host axons: (1) regenerated through the graft, (2) became ensheathed and myelinated by donor Schwann cells and (3) reinnervated denervated muscles. However, if tolerance was abolished, nerve allograft rejection occurred; and host axons in the graft degenerated and muscle again became denervated. Despite this unfortunate result, it was observed that host axons began a second regenerative effort into the remnants of the nerve allograft. We have now conducted a study to evaluate the outcome of this second regenerative response. A second study involved using the new immunosuppressive drug Cyclosporin A in which the key question asked was what happens to a nerve allograft if the drug prevents rejection but then is withdrawn. Our third study attempted to verify the report that freezing nerve allografts containing only minor transplantation antigens altered the graft so that after insertion into the host, successful axonal growth occurred through it over long distances. The author of that study also suggested that freezing the nerve allograft might induce a form of tolerance which was tested for in our study by regrafting recipients of frozen nerve allografts.

Methods Employed:

A) Tolerant study: Lewis (LE) rats were made tolerant to transplantation antigens of Brown-Norway (BN) rats by injecting the LE rats when newborn with bone marrow cells taken from (LEXBN)F<sub>1</sub> donors. This treatment causes suppressor lymphocytes to develop toward BN antigens with the result that any subsequent BN graft is accepted. When adult, the tolerant LE rats were given a 4 cm BN nerve graft which was joined to the cut, proximal or regenerative end of the host peroneal nerve. Three months later the tolerant rats were injected with LE spleen cells that were obtained from normal LE hosts which rejected BN skin. The injected sensitized LE cells can overpower the suppressor cells of tolerant hosts and cause, as they did, nerve allograft rejection and host nerve fiber degeneration. Having reached this point, the present study sought to determine if a second regrowth of host axons would occur through the remnants of a once successful nerve allografts in tolerance-abolished rats.

B) Cyclosporin A study: Normal LE rats were bilaterally grafted with 4 cm LE and BN nerve grafts; some went untreated while others received Cyclosporin A (Cs-A) 15 mg/kg/s.c. A third group of LE rats got bilateral BN nerve grafts and Cs-A. The nerve grafts were examined histologically, 30 days after grafting, for the presence of donor Schwann cells, a cellular immune reaction and presence of regenerated host axons. Only one BN nerve graft was removed at 30 days from Cs-A treated LE rats that received bilateral BN nerves. At that time Cs-A therapy was stopped in order to see what would happen to the second BN nerve graft. This experimental design was performed since it has been reported that short-term Cs-A treatment could induce a form of immunological tolerance.

C) Frozen Nerve Allograft study: Fischer (FR) rats received frozen LE nerve grafts which express only minor transplantation antigens. The LE nerve grafts were first placed in standard tissue culture medium, frozen gradually in a freezer set to -70°C, stored from 3 days to 6 weeks, thawed in a water bath at 37°C and finally placed in culture medium at room temperature to await transplantation. Isografts (grafts between genetically identical members of the same species which do not evoke an immune response) of nerve were similarly frozen and grafted. Nerve grafts were histologically examined 3-9 months later as described. In addition, rats with an isograft or allograft were regrafted with a fresh nerve isograft or allograft in order to determine if the frozen nerves sensitized the host. If sensitization occurred, rapid rejection of the fresh allograft should occur. However, if freezing the allograft produced tolerance, the second allograft should survive.

#### Major Findings:

A) Tolerance study: Host axons did not regenerate in any significant way into the remnants of a once successful nerve allograft in tolerance-abolished hosts. The number of axons traversing the graft was as poor as that found in a normal rat which actively rejected the allograft.

B) Cyclosporin A study: This immunosuppressive agent prevented rejection of all nerve allografts after 30 days of therapy. Untreated rats rejected the nerve allograft whereas Cs-A treated rats exhibited a graft similar to an isograft. The successful isograft or Cs-A treated allograft contained numerous Schwann cells, no immune cellular reaction was present and host axons regenerated into the graft. Unfortunately no tolerant state was induced after cessation of CS-A treatment since BN nerve grafts were ultimately rejected in rats that received bilateral allografts.

C) Frozen Nerve Allograft study: Frozen nerve grafts were unsuccessful regardless of whether they were isografts or allografts. In both cases very few host axons regenerated into the graft and no significant cellular reaction was noted. In addition, fresh allografts were rejected after they were placed in rats which had a frozen allograft demonstrating that no tolerant state was induced.



Significance:

A) Tolerant study: This result shows that continued immunosuppression is required to maintain a functional nerve allograft and that axons will not regenerate over any significant distance if the matrix they encounter is inert and devoid of Schwann cells.

B) Cyclosporin A study: This drug prevented the rejection of nerve allografts thereby permitting host axons to grow through them. However, if Cs-A therapy was terminated, rejection occurred indicating that the drug did not induce a state of tolerance. It will be necessary to reduce the dosage of Cs-A since after daily treatment with 15 mg/kg rats became inactive and lost weight (i.e., this dose produces toxicity).

C) Frozen Nerve Allograft study: Frozen isografts or allografts of nerve did not permit the passage of regenerating host axons. This suggests that viable Schwann cells are needed if a nerve graft is to be successful. Further studies are needed to determine what the Schwann cells provide. Perhaps living Schwann cells secrete a growth factor which encourages axonal growth. Freezing probably kills the Schwann cells in a graft and while this might reduce the cellular reaction to a graft, it has the side effect of eliminating the cell responsible for promoting axonal growth. The rejection of a second allograft in rats that had received a frozen allograft clearly demonstrates that no state of tolerance was achieved by this procedure. Our results conflict therefore with a previous report that advocated using frozen nerve allografts bearing only minor antigens to repair nerve tissue.

Proposed Course of Project:

A) Tolerant study: This project is complete and no further studies are contemplated except to look at early periods and determine how the injected sensitized cells caused graft rejection.

B) Cyclosporin A study: Long-term studies with reduced dosage of Cs-A (perhaps combined with other immunosuppressive agents) will be performed on nerve allografts. We will also determine whether nerve allografts with only minor antigens respond differently (i.e., survive) after cessation of Cs-A. Studies will also be done to see how allogenic neurons and skeletal muscle respond to Cs-A during and after stopping treatment.

C) Frozen Nerve Allograft study: This experiment is complete and no further studies are planned.

D) A series of studies will be undertaken to determine whether altering antigenicity affects the host's immune response to it. For example, nerve allografts will be treated with alloantibody before grafting in hope that this will mask the transplantation antigens. Allografts from neonatal donors will be evaluated for their reported effect of inducing some form of tolerance to a subsequent adult allograft of the same genotype.



Publications:

1. Zalewski, A. A., and A. K. Gulati. Survival of nerve and Schwann cells in allografts after Cyclosporin A treatment. Exp. Neurol. 70:219-225, 1980.
2. Zalewski, A. A., and A. K. Gulati. Rejection of nerve allografts after cessation of immunosuppression with Cyclosporin A. Transplantation 31:88-89. 1981.
3. Zalewski, A. A., A. K. Gulati, and W. K. Silvers. Loss of host axons in nerve allografts after abolishing immunological tolerance in rats. Exp. Neurol. 72:502-506, 1981.



Project Description:

Objectives: To determine the areas in the retina of greatest flux in energy metabolites and thus of greatest activity as a function of light/dark exposure. To determine the role, if any, of energy metabolites, cyclic nucleotides, phosphodiesterase, guanylate cyclase and calmodulin in the transduction process.

The study of granular dystrophy of the cornea is aimed at gaining a better understanding into the mechanism of inborn error of metabolism. Many hereditary anomalies of the cornea have been reported but their biochemical mechanisms are not well understood. The autosomal linked dystrophy of the cornea, known as granular dystrophy, is being studied. Granular dystrophy is characterized by an accumulation of granular particles in the stroma of the cornea. The nature of these particles which can eventually impair the vision of the patient is not at all understood; and much less is known about the mechanism leading to the formation of these granules in the cornea. It is hoped that by studying the biochemical mechanism of this disease it can lead us to better understanding of the mechanism of a whole class of hereditary disease associated with the cornea, and eventually lead to their treatment and prevention.

Methods Employed: Frogs are frozen whole in liquid nitrogen after either dark adaptation or light exposure, then stored until use at  $-70^{\circ}\text{C}$ . The eyes are removed in a cryostat, at  $-25^{\circ}\text{C}$ , mounted in a holder and  $6\text{ }\mu\text{M}$  sections cut at  $-22^{\circ}\text{C}$ . The sections are vacuum dried at  $-40^{\circ}\text{C}$  where they remain until use. For microdissection and analyses, the samples are warmed to room temperature still under vacuum and retinal layers dissected and weighed on quartz fiber balances. Microchemical analyses are performed in "pil wells" using volumes of  $0.05$  to  $5\text{ }\mu\text{l}$ , and capable of measuring  $10^{-15}$  mole of metabolites.

The first step of the project on granular dystrophy of the cornea is to analyze and determine the biochemical nature of the granules found in the cornea of the patients. Biopsies or diseased cornea from cornea transplantations are analyzed according to methods described below.

A) Histological study: Slides are prepared from cornea biopsies for verification and localization of the granules. Area of high concentration of granules are separated for biochemical analysis.

B) Polyacrylamide gel electrophoresis: Samples are solubilized with SDS and extracted for electrophoresis in slab gel developed according to established method.

C) Immunological study: Fluorescent antibodies and monoclonal antibodies against various known constituents of the cornea are used to verify the protein component of the granules.

D) Electron microscopy: Samples of the diseased cornea are also subjected to various electromicroscopic examination including elemental microanalysis.

Major Findings: In the study of light and dark adaptation in the frog retina, ATP and ADP has been measured in samples of 12 to 50 mg. The levels of ATP + ADP are remarkably similar in both light and dark adapted frogs ranging from 7 mmoles/kg in the pigment epithelium to 3 mmoles/kg in the outer segments, from where they rise steadily to a peak in 16 mmoles/kg in the inner nuclear layer. The 2 sec light exposure eyes have either the same value or are slightly higher than either the dark or 2 min eyes. These findings lend evidence to the hypothesis that light is the absence of stimulus for the  $\text{Na}^+$  current and demand for ATP decreases, at least transiently, especially in these cell layers.

The results obtained from the various studies on granular dystrophy of the cornea can be summarized as follows:

- 1) From gel electrophoresis, it was found that cornea with granular dystrophy has at least two protein components not found in normal cornea. One of these proteins has electrophoretic mobility which very closely resembles that of a keratin of molecular weight around 65,000 dalton.
- 2) From scanning electromicroscopic elemental analysis, the granules show unusually high level of sulfur-containing materials.
- 3) From immunological studies, negative results were obtained from staining with antibodies raised against various keratins component of the cornea epithelium.

Significance: The transduction mechanism for the action of light remains unknown, e.e., the steps between the absorption of light by rhodopsin and the generation of a nerve impulse. The involvement of a  $\text{Na}^+$  channel and changes in  $\text{Ca}^{2+}$  concentration have both been implicated in this process. Since the  $\text{Na}^+$  pump is a great user of energy, measuring changes in high energy compounds is one approach of looking at the energy requirements of this process. Calcium stimulation of enzymes is often mediated through calmodulin. A knowledge of its distribution might provide clues to its function in retina.

From the results on research on granular dystrophy of the cornea, it appears that the granules might be composed of a protein or proteins of high sulfur content which is immunologically distinct from the normal high sulfur-containing keratin found in the cornea. The fact that the appearance of these granules does not occur until the patients are well into adulthood suggests that the metabolic changes in the cornea continue well after differentiation has been completed during infancy. It is hoped that this study will help clarify some important aspect of biochemical differentiation of the cornea.

Proposed Course of Project: In the light- and dark-adapted frog retina ATP, P-creatine and AMP concentrations will be measured. The latter is a much more sensitive indicator of changes in adenylate nucleotide levels than either ATP or ADP and preliminary data indicate that dramatic changes will be seen in this metabolite.

An assay for calmodulin is planned which will be based on its stimulation of phosphodiesterase, and which will be sensitive enough to measure calmodulin levels in retinal layers. The AMP produced from the phosphodiesterase (first level of amplification) can be cycled using our modified ATP/ADP cycle to give at least an additional 4000-fold increase in sensitivity.

Publications:

1. Barbehenn, E., Masterson, E., Passonneau, J. and Chader, G.: Glutamine: A potentially important energy source in cultured pigment epithelial cells. Invest. Ophthalmol. Vis. Sci. 20 (ARVO Suppl.) 214 (1981).
2. de Azeredo, F.A.M., Lust, W.D. and Passonneau, J.V.: Light-induced changes in energy metabolites, guanine nucleotides, and guanylate cyclase within frog retinal layers. J. Biol. Chem. 256: 2731-2735, 1981.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02455-01 LNC
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Metabolic Correlates of Neuronal Transmission in the Hippocampal Slice.		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Janet V. Passonneau Other: Tim S. Whittingham Wesley D. Lust Hajime Arai Alexander B. Wheaton	Chief Staff Fellow Head, Sec. Neurochem. Pharm. Visiting Scientist Biol. Lab. Tech. (Micro)	LNC NINCDS LNC NINCDS LNC NINCDS LNC NINCDS LNC NINCDS
COOPERATING UNITS (if any)  None		
LAB/BRANCH Laboratory of Neurochemistry		
SECTION Section on Cellular Neurochemistry		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.5	PROFESSIONAL: 1.4	OTHER: 0.1
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The relationship between cellular metabolite levels and neuronal transmission is being investigated in the <u>in vitro hippocampus</u> . Currently, the concentrations of the <u>adenylates</u> , <u>phosphocreatine</u> , <u>creatine</u> , <u>lactate</u> , <u>GABA</u> and the <u>cyclic nucleotides</u> are evaluated for slices during <u>in vivo</u> and <u>in vitro</u> <u>ischemia</u> (up to 15 minutes) or an ensuing <u>in vitro</u> <u>recovery</u> period. The magnitude of the <u>evoked field potential</u> is also recorded. There is, as yet, no clear relationship between the recovery of neuronal transmission and the return to steady-state of any of the metabolites following ischemia. Of additional interest is the fall in total <u>adenylate</u> levels, <u>energy charge</u> and <u>phosphocreatine in vitro</u> compared to <u>in vivo</u> levels. Thus, the initial decapitation-induced <u>ischemia</u> irreversibly alters the metabolic profile of the <u>in vitro</u> hippocampus. The presence of the evoked response indicates that these marked changes are compatible with neural function. Subsequent <u>in vitro</u> <u>ischemic</u> episodes produce transient changes in the metabolite levels which are reversible to the new steady-state distribution. These <u>in vitro</u> <u>ischemic</u> changes are compared to those observed <u>in vivo</u> .		

## Project Description:

Objectives: To investigate the relationship between metabolite levels (particularly the cyclic nucleotides and those compounds associated with cellular energetics) and neuronal transmission during ischemia and anoxia. To determine if a perturbation in the level of a particular metabolite results in the loss of electrical function, and if recovery to steady-state of that compound corresponds to the return of electrical activity. Further, to manipulate the rate of change of the metabolite level in an attempt to increase the tissue's ability to survive paroxysmal ischemic or anoxic episodes.

To compare the metabolic profile of the in vitro hippocampal slice to that of the in vivo hippocampus. To modify the observed differences so that the in vitro hippocampus more accurately reflects the in vivo metabolic distributions.

Methods Employed: Adult male guinea pigs were decapitated and the hippocampi removed. ~ 500  $\mu$ m thick slices were cut freehand with a razorblade. Ischemic samples were taken for up to 15 minutes following decapitation. The remaining slices were suspended on a nylon mesh and superfused with standard bicarbonate Krebs' solution containing 4 mM glucose and equilibrated with 95% O<sub>2</sub>: 5% CO<sub>2</sub>. Additional samples were removed during this recovery period, which lasted up to 33 minutes. Some slices were exposed to a second, in vitro, ischemia by superfusing them with medium containing no glucose and equilibrated with 95% N<sub>2</sub>: 5% CO<sub>2</sub>. This was followed by a second recovery period. All tissue samples were immediately frozen by immersion and stirring in liquid nitrogen.

Evoked field potentials were obtained by 1 ppm bipolar stimulation of the perforant path axons. The tungsten recording electrode (2-3 m  $\Omega$ ) was placed in the region containing dentate granule cell somas.

In vivo metabolite levels were obtained by funnel freezing guinea pigs which were anesthetized with phenobarbital. The guinea pig continues to breathe spontaneously for 3 to 5 minutes during this process, resulting in the continued flow of oxygenated blood to the as yet unfrozen areas of the brain. The frozen samples of hippocampus were removed in a -20° C cryostat.

All tissue samples were extracted in perchloric acid, followed by enzymatic determination of ATP, ADP, AMP, phosphocreatine, lactate, GABA and pyruvate. Cyclic nucleotides (cAMP and cGMP) were measured by radioimmunoassay.

Major Findings: Experiments in this study have identified the changes which occur in the previously mentioned metabolites during in vivo ischemia and in vitro recovery. Furthermore, these changes are compared to an in vitro model of ischemia. Preliminary results indicate that normoxic in vitro slices exhibit a different metabolic profile from in vivo hippocampal tissue. Notably, the slices contain lowered levels of ATP, phosphocreatine (PCr) and total adenylates (by ~ 60%) as well as a decreased energy charge

$((\text{ATP} + 0.5 \text{ ADP})/(\text{ATP}+\text{ADP}+\text{AMP}) = 0.75)$ . The in vivo and in vitro ischemic models led to similar changes in metabolite levels both temporally and quantitatively. There was an initial rapid loss of PCr followed by a delayed, more gradual decline in ATP. That the drop in ATP is being retarded by myokinase activity is suggested by the rise in AMP secondary to a fall in ATP and concomitant increase in ADP. Lactate levels also increased dramatically during the in vivo ischemia, however, did not change in vitro (perhaps as the result of washout by the superfusate). Intracellular pH (as calculated from the creatine phosphokinase equilibrium) fell by  $\sim 0.5$  units within two minutes of ischemia both in vivo and in vitro, and recovered to near steady-state levels within five minutes of "reflow".

cAMP and cGMP levels remained stable or fell slightly during ischemia, and during the recovery period peaked at 100x (cAMP) and 30x (cGMP) ischemic levels. GABA levels also rose during recovery. The magnitude of the ischemic changes was not increased by extending the ischemic period from 7 to 15 minutes. ATP, PCr, pH, and energy charge all reached minimal levels within 7 minutes. However, the rate of recovery of the metabolic profile and the evoked response were both compromised by the 15 minute ischemia. There was no clear correlation between a particular metabolite level and failure (and recovery) of the evoked response. Previous work has indicated that critical changes in metabolite levels may occur in sub-cellular pools, and be masked by the greater bulk of the tissue (Lipton and Whittingham, in press). Thus, sampling more discrete regions of the hippocampal slice may demonstrate a metabolic dependence of electrical transmission during ischemia and recovery.

Significance to Biomedical Research and the Institute Program: This study will define the metabolic changes which occur during the preparation and subsequent incubation of hippocampal slices. Given the large number of projects currently using the hippocampal slice, it will be beneficial to know how this preparation compares to its in vivo correlate. It may then be possible to slightly alter the standard incubation solution in order to have the metabolic profile of the in vitro slice better approximate that of the in situ hippocampus.

The relationship between metabolite levels (cAMP, cGMP, and the high energy phosphates) and neuronal transmission may be of major importance. Methods will be tested which may result in increased viability of mammalian neural tissue during short to longer term ischemic and hypoxic episodes. The in vitro preparation also allows for more controlled investigations of the interrelationship of energy production and neural function than have previous in situ studies.

Proposed Course of Project: This study has defined a number of metabolic perturbations which occur in whole slices during in vivo and in vitro ischemia, as well as during an in vitro recovery period. In association with these studies, an attempt will be made to bolster high energy phosphate reserves (both PCr and ATP) by several methods.

Previous work (Lipton and Whittingham, in press) has indicated the dependence of electrical activity on ATP levels within defined areas of the hippocampal slice. Additional experiments will identify more localized metabolic pools within the slice, particularly in the region of the synapse between perforant path axons and dentate granule cells. This might be accomplished by locating the sub-cellular distribution of the enzymes associated with maintenance of ATP levels (specifically, creatine phosphokinase and hexokinase). In addition, intracellular recordings will be taken from dentate granule cells in order to better define the changes in electrical characteristics of mammalian neurons during ischemia and anoxia.

Publications: None.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02257-05 LNC												
PERIOD COVERED October 1, 1980 to September 30, 1981														
TITLE OF PROJECT (80 characters or less)  Neuropharmacology of Cerebral Metabolism														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">W. D. Lust</td> <td style="width: 35%;">Head, Sect. Neurochem. Pharm.</td> <td style="width: 15%;">LNC NINCDS</td> </tr> <tr> <td>Other:</td> <td>J. V. Passonneau</td> <td>Head, Sect. Cell. Neurochem.</td> <td>LNC NINCDS</td> </tr> <tr> <td></td> <td>A. B. Wheaton</td> <td>Biol. Lab. Tech. (Micro)</td> <td>LNC NINCDS</td> </tr> </table>			PI:	W. D. Lust	Head, Sect. Neurochem. Pharm.	LNC NINCDS	Other:	J. V. Passonneau	Head, Sect. Cell. Neurochem.	LNC NINCDS		A. B. Wheaton	Biol. Lab. Tech. (Micro)	LNC NINCDS
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	A. B. Wheaton	Biol. Lab. Tech. (Micro)	LNC NINCDS											
COOPERATING UNITS (if any) Pharmacology Laboratory, Epilepsy Branch, NDP, NINCDS, (Bldg. 36)														
LAB/BRANCH Lab. of Neurochemistry														
SECTION Section on Neurochemical Pharmacology														
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TOTAL MANYEARS: 0.7	PROFESSIONAL: 0.3	OTHER: 0.4												
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS														
SUMMARY OF WORK (200 words or less - underline keywords) It is increasingly evident that 1) the rate of energy consumption is coupled to CNS function and 2) the <u>cyclic nucleotides</u> as neuro-effectors may play an important role in neuronal excitability. Therefore, any changes in the brain levels of the cyclic nucleotides and the <u>energy metabolites</u> may reflect alterations in neural function. These parameters were examined after treatment with the following neurotropic agents: 1) <u>anticonvulsants</u> , 2) <u>convulsants</u> and 3) anesthetics. Major shifts in the balance between energy production and energy utilization were only evident during overt convulsive behavior. The anesthetic, halothane, also affected the profile of the energy metabolites, however the energy status of the tissue was maintained. Cyclic GMP was affected by all three classes of agents, whereas cyclic AMP only changed during the excitable stages of convulsion. These parameters are presently being examined in brains from 'kindled' rats.														



Objectives: a) To determine what effect the different groups of neurotropic agents have on energy metabolism and on cyclic nucleotide metabolism.

b) To investigate the role of the metabolite changes to the initiation, propagation and termination of experimental seizures.

Methods employed: Animals were rapidly frozen either intact or in situ with liquid nitrogen at the appropriate times after treatment. The brains were removed at  $-25^{\circ}$ , weighed and extracted in perchloric acid. The neutralized extracts were used in all subsequent assays. When discrete areas of the brain were examined, the tissue was sectioned at  $-20^{\circ}$ , dried at  $-45^{\circ}$ , and the brain area free-hand dissected at room temperature. The tissue was weighed on a quartz-fiber balance and extracted in either sodium hydroxide or hydrochloric acid depending on the metabolite to be measured.

The cyclic nucleotides were measured by radioimmunoassay and the energy metabolites were determined by direct enzymatic analysis for regional studies. Studies on discrete layers within a region required the use of enzymic cycling.

Maximal electroshock was applied by corneal electrodes at an intensity of 50 mA for 0.2 sec. Amygdala kindling was achieved by placing the electrodes in the right amygdala and stimulating this region daily at a set time and intensity until a full blown seizure was elicited from a single stimulation.

Major findings: Experimental seizures. The studies on maximal electroshock (MES) clearly demonstrated that during the excitable stages of convulsion that there was a significant depression of the energy status of the tissue and an increase in the concentrations of both cyclic nucleotides. The increase in cyclic AMP was far greater than that for cyclic GMP. A comparison of the responses in the cerebellum (C) and the cerebral cortex (X) indicated that the magnitude and the duration of the cyclic AMP response in the C was substantially less than in the X. Since the cerebellum is thought to have an inhibitory influence on other regions of the brain and further that cyclic AMP has an inhibitory effect on the output of the cerebellum, the elevation of the cyclic AMP in the C may be critical to the expression of the seizure. In fact, the anticonvulsant, phenytoin, partially prevents the increase in cerebellar cyclic AMP and also prevents the tonic extensor phase of the seizure. It is quite possible that if normal cerebellar output was maintained during convulsions, the severity of the seizure would be reduced.

Another interesting aspect of the metabolite response in the C and X is the observation that the levels of high-energy phosphates in the C were approximately 25% higher than in the X. A similar difference exists between single Purkinje cells of the C and single pyramidal tract neurons of the X. The question arises is there a relationship between the concentration of the energy reserves and the magnitude of the cyclic AMP response? Because of the higher energy reserves in the C, the depression of ATP during seizures is less in the C. Therefore, less 5'AMP and adenosine would be produced. Since adenosine is a potent stimulus to the synthesis of

cyclic AMP, it would be expected that the cyclic AMP increase would be less than in the X, which it is. Therefore, there does appear to be an association between energy status and the cyclic AMP produced during seizures. Another approach to this problem is to experimentally increase the energy reserves of the tissue and then challenge the animals with MES. It has been shown that cyclocreatine when given in the diet over a long period of time forms cyclocreatine-P in the brain, effectively doubling the energy reserves. Preliminary experiments indicate that such a treatment does affect the seizure response, however little or no differences in cyclic AMP levels after MES were observed between treated and control groups. One major problem is that cyclocreatine-P is not mobilized as rapidly as P-creatine. In spite of this, further experiments are being performed to clarify the preliminary reports.

Since there are obvious drawbacks to the use of MES as a model of experimental seizures, a kindling model has been developed. Initially, the emphasis has been to measure the levels of ATP, P-creatine, GABA and the cyclic nucleotides in the amygdala, hippocampus and cortex in control and kindled animals. Thus far, it does not appear that the steady-state levels of these metabolites are affected. This, however, is a necessary first step to studying the effects of both convulsants and anticonvulsants on a seizure focus and those regions secondarily affected by it.

#### Significance to Biomedical Research and the Institute Program.

A convulsion is a symptom of an underlying abnormality in brain function. Efforts to identify the cause of the spontaneous uncontrolled discharge of the neurons have indicated that the process leading up to and triggering the seizure is quite complex. Besides evaluating the changes that reduce the seizure threshold, other events occurring during the propagation and termination of the convulsion are also of interest, since these processes may be equally important in the control of seizures.

The evidence to date indicate that of all the metabolites examined only cyclic GMP and GABA appear to play a role in the onset of seizures. The depletion of high-energy metabolites and the increase of cyclic AMP seem to be a result of seizure activity. While this tends to rule out their involvement in seizure susceptibility, it is quite possible that these changes play a role in the termination of overt seizures. The significance of the cyclic GMP to the triggering of seizures is supported by the finding that anticonvulsants generally prevent the preconvulsive rise in cyclic GMP as well as the seizure itself. Pharmacological manipulation of the cyclic GMP system may be a useful criterion for evaluating therapeutic efficacy.

Proposed Course of Project: Using the kindling model, the goal is to determine if the metabolism of GABA or the cyclic nucleotides as well as energy metabolism is perturbed at the seizure focus. Eventually, these metabolites will be examined at the onset and during the propagation of an induced seizure.

In subsequent investigations on neurotropic drugs as well as seizures, a greater emphasis will be placed on the discrete sampling of the tissue. It has been our experience that a number of phenomena only become evident after microsampling.

Publications:

Lust, W.D., Feussner, G.K., Passonneau, J.V. and McCandless, D.W.: Biochemical mechanism of anticonvulsants: Studies on cyclic nucleotide systems in brain. In (Palmer, G., ed.): Neuropharmacology of Central Nervous System and Behavior Disorders. New York, Academic Press, 1980, pp. 407-430.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02429-02 LNC										
PERIOD COVERED October 1, 1980 to September 30, 1981												
TITLE OF PROJECT (80 characters or less)  Coordinate Effects of Amphetamine on Brain Energy Metabolism and Protein Synthesis												
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI:</td> <td style="width: 33%;">Janet V. Passonneau</td> <td style="width: 33%;">Chief</td> <td style="width: 33%;">LNC</td> <td style="width: 33%;">NINCDS</td> </tr> <tr> <td>Other:</td> <td>Thaddeus S. Nowak, Jr.</td> <td>Staff Fellow</td> <td>LNC</td> <td>NINCDS</td> </tr> </table>			PI:	Janet V. Passonneau	Chief	LNC	NINCDS	Other:	Thaddeus S. Nowak, Jr.	Staff Fellow	LNC	NINCDS
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TOTAL MANYEARS: 1.2	PROFESSIONAL: 1.2	OTHER: 0										
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SUMMARY OF WORK (200 words or less - underline keywords)  <p>           Effects of <u>amphetamine</u> on <u>brain energy metabolism</u> and <u>protein synthesis</u> are being investigated under conditions which give rise to changes in <u>body temperature</u> in response to the drug. In particular, metabolic changes associated with drug induced <u>hyperthermia</u> are being correlated with the <u>hyperthermia-dependent inhibition of brain protein synthesis</u> by <u>amphetamine</u>. Effects on protein synthesis are determined by means of <u>brain polyribosome profiles</u>. Metabolites of interest include <u>brain glucose</u>, <u>glycogen</u> and <u>high-energy phosphates</u>, especially <u>guanine nucleotides</u>, which are measured enzymatically. <u>Hyperthermia-associated effects</u> on all of the above metabolites have been demonstrated following <u>amphetamine administration</u>. The previously observed <u>brain glycogenolysis</u> induced by <u>amphetamine</u> has now been found to be <u>correlated with hyperthermia</u> in response to the drug. Significant <u>reductions in brain GTP levels</u> during <u>amphetamine-induced hyperthermia</u> may be responsible in part for the inhibition of brain protein synthesis which is also observed.         </p>												



Project Description:

Objectives: To determine whether known effects of amphetamine on brain energy metabolism (e.g., increased glycogen breakdown) are correlated with hyperthermia produced by the drug, and to clarify the mechanism by which amphetamine induces glycogenolysis. To characterize other possible changes in brain metabolites, particularly guanine nucleotides, during amphetamine-induced hyperthermia. To correlate these effects of amphetamine on brain metabolites with the inhibition of brain protein synthesis by amphetamine, which is known to involve a hyperthermic response to the drug, and thereby to elucidate possible biochemical control mechanisms by which energy status regulates protein synthesis.

Methods Employed: d-Amphetamine sulfate is administered by intraperitoneal injection to mice housed at various temperatures. (An ambient temperature of 27° C. is used in routine studies requiring amphetamine-induced hyperthermia). At appropriate times after injection the mice are frozen rapidly in liquid nitrogen, and samples of cerebral and cerebellar cortex are assayed for glucose, glycogen, adenine and guanine nucleotides, and other metabolites using enzymatic assay methods. Polyribosomes are prepared from the remaining brain tissue, which are further fractionated on sucrose gradients to yield "polyribosome profiles" as an index of brain protein synthesis.

Major Findings: We have established that the well-known glycogenolytic action of amphetamine in brain is, in fact, associated with drug-induced hyperthermia, which has previously been shown to be the case for the inhibition of brain protein synthesis by this drug. Small (20%) decreases in brain ATP and phosphocreatine levels are also observed under conditions of drug-induced hyperthermia, along with a 20% increase in brain cAMP. Notably, brain GTP is more markedly reduced (by 35%) and, since GDP and GMP levels do not change, total guanine nucleotide levels fall. The above changes in metabolite levels are not observed under conditions of ambient temperature which attenuate the amphetamine-induced hyperthermia. The effect of amphetamine on brain GTP levels thus may be at least in part responsible for the inhibition of brain protein synthesis under conditions of hyperthermia. In any case, the above observations demonstrate that amphetamine-induced hyperthermia provides a useful model for the study of the coordinate regulation of brain energy metabolism and protein synthesis.

Significance to Biomedical Research and the Program of the Institute: These studies should provide information about the functional, physiological role of catecholamine pathways in brain, as characterized by their sensitivity to the pharmacological actions of amphetamine. On a fundamental biochemical level they should help to characterize the control mechanisms by which energy metabolism must be linked with protein synthesis and other energy-utilizing cellular processes. Since there are other experimental treatments of neurological interest which simultaneously affect brain energy metabolism and protein synthesis (e.g., electroconvulsive shock, convulsant drugs, spreading depression, transient ischemia), these studies may help to establish a



common metabolic response to these various interventions which might be responsible for the effect on protein synthesis.

Proposed Course of Project: A method is under development for assessing the status of brain protein synthesis by the use of in vitro incorporation of radioactive amino acids, as a replacement for the somewhat cumbersome polysome profiles, which will then be applied to studies of amphetamine effects on this process. The enzymes regulating glycogen synthesis and breakdown (glycogen synthetase and phosphorylase, and their respective kinases and phosphatases) will be measured following amphetamine administration under conditions which allow, and fail to allow, drug-induced hyperthermia, in order to clarify the mechanism of the drug's glycogenolytic effect. The studies of amphetamine-induced changes in brain guanine nucleotide metabolism will continue with emphasis on determining the mechanism by which GTP and total measurable guanylate levels are reduced by the drug. This will be approached primarily with the use of high performance liquid chromatography to measure levels of metabolites in the degradation pathway for purine nucleotides and to look for the possible appearance of novel nucleotides derived from guanylates which are not measured by the enzymatic assays used to date. In all of the above studies of brain protein synthesis and energy metabolism additional experiments will be included using hyperthermia produced by direct heating of the animals, in order to dissociate those effects due to the drug from those which might be mediated by the elevation of body temperature per se.

Publications: None.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02430-02 LNC												
PERIOD COVERED October 1, 1980 to September 30, 1981														
TITLE OF PROJECT (80 characters or less) Aspects of Calcium Metabolism in Electric Tissue														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT														
<table style="width: 100%; border: none;"> <tr> <td style="width: 10%;">PI:</td> <td style="width: 35%;">Robert W. Albers</td> <td style="width: 35%;">Head, Sect. on Enzyme Chemistry</td> <td style="width: 20%;">LNC NINCDS</td> </tr> <tr> <td>OTHER:</td> <td>Stephen P Chock</td> <td>Research Assoc.</td> <td>LNC NINCDS</td> </tr> <tr> <td></td> <td>Lynn M. Amende</td> <td>NRSA, Enzyme Chemistry</td> <td>LNC NINCDS</td> </tr> </table>			PI:	Robert W. Albers	Head, Sect. on Enzyme Chemistry	LNC NINCDS	OTHER:	Stephen P Chock	Research Assoc.	LNC NINCDS		Lynn M. Amende	NRSA, Enzyme Chemistry	LNC NINCDS
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	Lynn M. Amende	NRSA, Enzyme Chemistry	LNC NINCDS											
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SECTION Section on Enzyme Chemistry														
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SUMMARY OF WORK (200 words or less - underline keywords)														
<p>Regulatory roles of calcium in Electrophorus electric organ are under investigation. The studies consist of two parts: (1) the isolation and purification of <u>calmodulin</u> from electric tissue, (2) isolation and characterization of a <u>Ca-ATPase</u> from electric tissue, and (3) investigation of the <u>interaction</u> of <u>calmodulin</u> with <u>electroplaque membrane components</u>.</p>														

Project Description:

Objective: These studies are designed to assess the role of calcium ions in the function of the highly specialized cholinergic tissue exemplified by the Electrophorus electric organ.

Methods Employed:

A) Calmodulin is assayed by a coupled spectrophotometric method newly developed in this laboratory. A new method for isolating calmodulin in high purity and high yield from electric organ tissue has been developed.

B) The Ca-ATPase is measured by phosphate analysis or by a coupled spectrophotometric assay. The enzyme is isolated by a newly developed procedure.

Major Findings:

A) The method of calmodulin assay has been improved in terms of quantitation and sensitivity. The new method of calmodulin purification provides large amounts of pure protein for characterization and for use in the ancillary studies. About 5% of the total soluble protein in the electric organ appears to be calmodulin. Yields of about 200 mg/kg of tissue are obtained.

B) A Ca-ATPase has for the first time been identified in the electric organ tissue. A method for solubilizing this enzyme from the cell membrane fraction has been developed and the enzyme has been further purified by use of a calmodulin affinity column.

Significance: Calmodulin is a ubiquitously distributed protein that is considered to mediate most of the intracellular actions of calcium ions. Electric organ tissue contains calmodulin in unusually high levels. Because of the clearly defined cholinergic function of this tissue it provides a unique opportunity to examine the role of calmodulin in cholinergic function.

Proposed Course of Project: Two of the investigators involved in this project will be leaving the section. The work on calmodulin and the Ca-ATPase will be completed and prepared for publication.

Publications:

None







# ANNUAL REPORT

October 1, 1980 through September 30, 1981

## Laboratory of Molecular Biology

National Institute of Neurological and Communicative Disorders  
and Stroke

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ANNUAL REPORT  
October 1, 1980 through September 30, 1981  
Laboratory of Molecular Biology  
National Institute of Neurological and Communicative  
Disorders and Stroke

Ernst Freese, Chief

The Laboratory has identified the molecule (GTP) whose decrease initiates bacterial sporulation as result of nutritional deprivation; it has found that similar starvation conditions cause the initiation of meiosis and the subsequent sporulation of yeast; it has observed that the lipophilic acid butyrate, which induces differentiation of mammalian cells, decreases the methylation of RNA; and it has quantitatively analyzed in mouse cultures the teratogenic effect of the two lipophilic acids valproate and diphenylhydantoin, two major antiepileptic drugs. The results demonstrate that our vertical approach to the study of differentiation is successful. Results found in bacteria can be used to predict results in eukaryotes such as yeast and mammalian cell cultures, and the latter can be used to anticipate developmental responses in mammals. The results will be presented in this sequence, starting with the simple and ending with the complex organisms.

1. Molecules controlling bacterial differentiation (sporulation). Microbial differentiation generally starts under conditions of nutritional deprivation. A similar nutritional control of differentiation must exist also in early embryogenesis and is known to exist during germ cell development, but the mechanisms of these processes have not been studied. The Laboratory has shown that sporulation of B. subtilis starts under conditions under which nutrition is scarce but not completely absent. Such conditions arise automatically at the end of exponential growth in a rich medium, and they can be artificially generated by the step-down transfer from a rich to a poor medium. Under such step-down conditions, a stringent response sets in which is accompanied by the production of large amounts of ppGpp and pppGppp and the decrease in the rates of RNA, protein, wall and membrane synthesis. A drastic stringent response can also be produced by the complete removal of an amino acid from an auxotrophic mutant, but the ensuing severe curtailment of metabolism does not lead to sporulation. Only when additional transport mutants or other conditions are used, which allow the manipulation of the intracellular concentration of amino acids by the extracellular concentration, is it possible to obtain sporulation at some intermediate concentration of the amino acid. Thus transient or partial but not complete stringent response initiates sporulation. The involvement of stringent response was proven by the fact that a relaxed mutant (relA) did not sporulate under any of these conditions.

The initiation of sporulation is not a direct consequence of the increase of ppGpp but results indirectly from the corresponding decrease of GTP. Sporulation can also be induced in the relaxed (rel) strain by any conditions which decrease the concentration of GTP (without increasing that of ppGpp). Such conditions can be obtained in the presence of excess glucose, nitrogen, and phosphate by the addition of inhibitors such as decoyinine or mycophenolic acid or by the use of guanine mutants.

It was known that certain antibiotics acting on ribosomes inhibit microbial differentiation without much affecting growth, and it was consequently assumed that certain properties of ribosomes, present or produced during development, were needed to allow synthesis of developmental proteins. We have shown that the effect of several of these antibiotics can be counteracted by the addition of decoyinine or by other methods causing the decrease in the synthesis of guanine nucleotides. Nucleotide measurements revealed that these antibiotics simply reduced the stringent response. These studies also showed that the inhibition by low concentrations of Kasugamycin could be specifically counteracted by thiamine but not by the (few) metabolites whose synthesis is controlled by thiamine pyrophosphate. The possibility that the antibiotic may enter the cell via a thiamine transport system is now under investigation. Other studies produced mutants for which a significant fraction enters differentiation at every generation while the cells multiply while growing exponentially. Genetic and biochemical studies determined that this phenomenon resulted from a partial deficiency in pyruvate carboxylase, this limits the supply of aspartate and glutamate and apparently causes sporulation by a slight stringent response. Whereas this slight development effect is barely noticeable in one generation, it eventually leads to differentiation of almost all (90%) cells.

In the attempt to determine how GTP deficiency initiates differentiation, the synthesis of all major polymers was measured under conditions of sporulation induction and under comparable conditions of no sporulation but equal growth reduction. The synthesis of RNA, especially that of rRNA was reduced under both conditions. Certain enzyme and transport changes were observed only under sporulation conditions but further studies will be necessary to determine whether they are required for sporulation. Whereas membrane synthesis was not affected, cell wall synthesis was rapidly reduced when the concentration of GTP decreased. This was surprising because guanine nucleotides are not known to affect cell wall synthesis at any intermediate or final step. This interesting control mechanism may be related to the asymmetric and maybe even to the normal division septation and will have to be further investigated.

B. subtilis, like most Gram positive organisms, has membrane phospholipids which are to about 70% branched. To examine whether a particular branched fatty acid had to be present in

large concentrations to allow growth or sporulation, we used a branched fatty acid mutant. This strain could grow and sporulate on many different branched or cyclic fatty acid precursors which were incorporated into the phospholipids. Thus it does not matter which branched fatty acid enables the membrane to maintain its flexibility. However, the fraction of branched fatty acid needed for growth is much lower than that needed for sporulation. Whereas vegetative membranes of the rod shaped bacteria can contain up to 75% straight-chain fatty acids, spores contain maximally 25% straight-chain fatty acids. Possibly, the round portions of the membrane, such as the cell ends or the whole forespore, require a higher percentage of branched fatty acids to be sufficiently flexible than the straight portions of the rod.

## 2. Mechanisms controlling meiosis and yeast sporulation.

Although the eukaryote *Saccharomyces cerevisiae* has been thoroughly studied biochemically and genetically, the above studies of identifying the compound controlling differentiation could not have been performed. Because the yeast cells sporulate only in the diploid ( $a \alpha$ ) form, extensive genetic manipulation of auxotrophic and transport mutants is necessary before the proper strains for analysis can be produced. In addition, yeast is extremely resistant to most inhibitors effective in bacteria which indicates that only few compounds of small molecular weight can pass the screen of the protective wall and only few compounds are actively transported. Nevertheless, an examination of yeast differentiation is most worthwhile because sporulation is preceded by meiosis whose biochemical mechanism is not known in any organism. The Laboratory has shown that yeast meiosis and sporulation, like that of *B. subtilis*, can be initiated by a partial, but not complete, deprivation of carbon, nitrogen, or phosphorus containing compounds in the presence of excess of all other nutrients needed for growth. Presumably, a nucleotide controls the onset of meiosis. In the attempt to identify this nucleotide further, the laboratory tested a number of inhibitors of nucleotide synthesis. The only effective inhibitor of yeast was hadacidin, which at very high concentrations (100 mM) induces sporulation in a medium in which no sporulation otherwise occurred. Hadacidin inhibits the synthesis of adenine nucleotides and thereby indirectly the synthesis of all nucleotides. The Laboratory has also isolated by genetic crosses one guanine requiring diploid; this strain sporulates extremely well upon partial removal of guanine from the medium. Although these results and the corresponding nucleotide measurements have to be checked in detail, they indicate that the meiosis of yeast (and thus presumably of all organisms) may be initiated by the deficiency of guanine nucleotides.

## 3. Development of receptors needed for intercellular communication. Several years ago, the Laboratory had shown that butyrate induces in HeLa cells various morphological changes and



increases the number of  $\beta$ -adrenergic receptors by a factor four. This finding has been very useful in analyzing the biochemical events of adenylate cyclase activation and the role of phospholipid methylation in this process. The Laboratory has now shown that in a pituitary tumor cell line both  $\beta$ -adrenergic and  $\alpha$ -adrenergic receptors can be increased by exposure to butyrate. This may enable the study of the relative and combined actions of these receptors whose occupancy by adrenalin has opposite effects on the cell. In the study of the mechanism whereby butyrate induces the production of receptor proteins, the Laboratory found that butyrate reduces the methylation of mRNA. It will now be determined whether this methylation change occurs only in the "cap" of mRNA or is distributed over the whole molecule. This may help to understand how specific developmental genes are induced. In other studies, it has been shown that the inhibition of DNA synthesis, normally observed in HeLa cells, is not necessary for the induction of receptor proteins because that still occurs in a mutant in which DNA synthesis is not inhibited.

Other lipophilic acids with pronounced effect on the CNS are the benzodiazepines such as valium. Receptors for these compounds occur both in the CNS and in peripheral tissue. The Laboratory has developed a technique whereby specific benzodiazepines can be used to differentiate between CNS type receptors, which are limited to neurons, and other receptors which are mainly found in peripheral tissue. This technique can now be used to selectively grow neurons in culture.

#### 4. Teratological studies in mouse embryo cultures.

Previous studies in the Laboratory had shown that the inhibition of bacterial and mammalian cell growth by lipophilic acids is roughly proportional to their octanol/water partition coefficient. Compounds with special actions such as butyrate or analgesics/antipyretics were more effective. In a higher organism, the correct rate of cell multiplication is probably necessary for normal development, e.g. for the closure of structures such as the palate or the neural tube. Therefore, it was predicted that the most effective inhibitors of individual cell growth would also have a teratological effect. This prediction was (to a limited extent) examined by teratogenic studies in whole mice; most of the predicted compounds were teratogenic. To avoid the difficulties of absorption, metabolism and excretion of compounds before they can reach the embryo, a mouse embryo culture system was then started in which compounds can be added directly to the culture medium (rat serum) in which the embryos develop. Embryos can be explanted on the ninth day of gestation and then maintained in a culture of rat serum, with all development being normal for as long as 48 hours. The developing embryos were examined under the stereo microscope and also by inspecting transverse sections under the light and the

electron microscope. These refined techniques enabled the detection of deficiencies that were not seen by gross microscopic observations.

Two compounds, valproic acid and diphenylhydantoin were examined in detail because they are both used as antiepileptic drugs. Quantitative studies clearly established a dose dependent teratogenic effect for either of these drugs and showed malformations of the heart, brain, spinal cord and cranio-facial structures. Several percent of malformations were observed at concentrations of the drugs found in the plasma of treated patients. The more lipophilic diphenylhydantoin had a higher teratogenic potency than valproic acid. It was remarkable that some teratogenic effects were detectable already at valproate concentrations eight-fold lower than those needed to inhibit fibroblast growth by 50%. Thus valproate either specifically affects certain embryonic cells or a very small inhibition of the growth of all cells can already induce teratogenicity.

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SUMMARY OF WORK (200 words or less - underline keywords) Partial stringent control of polymer synthesis, caused by partial amino acid deprivation, initiates bacterial sporulation. It is accompanied by increase of ppGpp and decrease of GTP. This was shown under step-down conditions from rich media and in auxotrophs deficient in active amino acid transport. Certain <u>antibiotics</u> prevent sporulation because they interfere with this stringent control. All studies showed that a decrease of GTP is associated with the initiation of sporulation. To determine the mechanism of GTP, RNase and enzyme synthesis, membrane synthesis and transport, and cell wall synthesis were studied. A continuously sporulating strain was genetically and biochemically analyzed and shown to be partially deficient in pyruvate carboxylase. The special need of <u>branched chain fatty acids</u> for growth and sporulation were also examined.																																										

Project Description:

Objectives: The differentiation of microorganisms and of certain cell types in higher organisms begins when rapidly metabolizable carbon or nitrogen sources or phosphates have been exhausted or when the cells are exposed to a sudden shift-down from a rich to a poor medium. Results in the Laboratory have established that the decrease of GTP is sufficient to initiate sporulation. To determine whether GTP or some other compound was responsible for sporulation during step-down or stringent control conditions, various methods were used to initiate partial stringent control. For this purpose special transport mutations had to be isolated. We also examined the mechanism whereby certain antibiotics prevented sporulation because we suspected that this did not reflect a necessary alteration of ribosomes during differentiation. To understand why the standard strain does not sporulate during growth, mutants were isolated which continuously sporulate at a significant frequency while the culture is growing, and the biochemical properties of these mutants were determined. Because all these results pointed to GTP controlling sporulation, the effect of GTP decrease on the synthesis of various cell polymers was studied both to determine which of the polymer changes were necessary for sporulation and to find specific changes that were always associated with sporulation. Because the composition of branched fatty acids in cell membranes can vary, the effects of such variations were studied in a branched fatty acid mutant.

Methods Employed: Nucleotides were measured both by  $^{32}\text{P}$  incorporation and thin layer chromatography and by high pressure chromatography of formic acid extracts. A number of mutants and conditions whereby the intracellular concentration of an amino acid could be controlled by the extracellular concentration of the compound. This was done in a methionine mutant by feeding D-methionine instead of L-methionine, in an isoleucine-valine auxotroph, by introducing a mutant defective in the transport of keto acids precursors, and in an aspartate requiring mutant, by saturating the high-affinity transport for aspartate and glutamate by excess glutamate.

Major Findings:

1. Initiation of sporulation by stringent control. Various step-down conditions, which have frequently been used to initiate sporulation, were examined to determine whether sporulation was due to stringent control. The properties of a stringently controlled ( $\text{rel}^-$ ) and a relaxed ( $\text{rel}^+$ ) strain were compared. It was found that the step-down not only from a medium containing all amino acids to one containing glutamate but also from a glucose to a lactate medium caused sporulation by stringent control. In order to see whether this effect was limited to



certain amino acids, amino acid auxotrophs for aspartate, isoleucine and valine, and methionine were individually used to initiate sporulation caused by amino acid deprivation. However, it was necessary in each case, to control the intracellular amino acid concentration by the extracellular supply, which was possible only under conditions under which the active transport of the amino acid was circumvented. Good sporulation was then observed at an intermediate amino acid concentration in the stringent but not the relaxed strain.

Under all these stringent conditions, the increase of ppGpp and pppGpp was correlated with a decrease of GTP whereas the other nucleotides increased under some and decreased under other conditions. To determine whether sporulation was due to the increase of ppGpp or the decrease of GTP, a mycophenolic acid resistant mutant was isolated in the isoleucine/valine auxotroph. This mutant still showed the increase of ppGpp but a reduced decrease of GTP, and it did not sporulate. Apparently, the inhibition of IMP dehydrogenase by ppGpp caused a decrease in the synthesis of guanine nucleotides and therefore caused sporulation. In the mycophenolic acid resistant mutant, this effect was reduced and sporulation could not occur.

Sporulation was due to the initial drastic decrease of GTP. Later, the cells adapted to the partial deficiency of an amino acid and resumed growth without any further initiation of sporulation as was shown in a long term continuous culture experiment.

Under all conditions, the relaxed mutant could be initiated to sporulate by the addition of decoyinine that is by the direct inhibition of the synthesis of guanine nucleotides without any increase of ppGpp or pppGpp.

2. Effects of antibiotics on sporulation. It was well known that certain antibiotics prevent sporulation at concentrations at which they have no or very little effect on growth. Because all these antibiotics bind to ribosomes, it was assumed that certain structures or changes of ribosomes were necessary for differentiation but not for growth. Our finding that most sporulation media cause sporulation by the stringent response suggested an alternate explanation of the antibiotic effect. Indeed, we found that the antibiotics kasugamycin, fusidic acid, and chloramphenicol, which completely prevent sporulation at low concentrations, were no longer effective if the medium also contained decoyinine causing a direct decrease of guanine nucleotides. Nucleotide measurements showed that addition of the antibiotic reduced the stringent response, i.e. the increase of ppGpp, and the corresponding decrease of GTP. Thus, there is no need to assume that ribosomes have to change in order to allow sporulation. In the course of these studies it was also found that kasugamycin at very low concentrations, had a



strong inhibitory effect which could be counteracted by adenine containing compounds and most effectively by thiamine. This effect suggests that kasugamycin may be taken up by the thiamine transport system and opens an interesting study into the mechanism of antibiotic resistance.

3. Sporulation in special mutants. In order to determine whether the initiation of sporulation by deprivation of guanine nucleotides was due to a decrease in GMP, GDP or GTP, a GMP reductase mutant was isolated and its sporulation properties were studied. If an additional mutation in the general purine pathway was introduced, the mutant could grow on hypoxanthine. But addition of guanosine reduced the rate of growth, and the synthesis of adenine nucleotides was inhibited due to competition of the intracellularly accumulated GMP for the synthesis of IMP from the extracellular hypoxanthine. Interestingly, the addition of guanosine initiated sporulation in this strain; it caused a decrease of GTP, obviously due to the decrease in ATP. Thus, it is clear that the sporulation induction is due to the decrease of GTP or GDP but not that of GMP.

In the course of these studies, mutants were observed which sporulated continuously in a minimal medium but no longer sporulated when compounds such as glutamate, aspartate or citrate cycle intermediates were added. By introducing additional mutations, this phenomenon was investigated and found to result from a decreased specific activity of pyruvate carboxylase, an enzyme needed to derive from glucose oxaloacetate and thus aspartate. The sporulation is therefore due to a continuous deficiency of aspartate. Whether this phenomenon is due to a mild stringent control or another effect of aspartate deficiency is now under investigation.

4. Other cellular consequences of a GTP decrease. A systematic study was launched to determine why the decrease of GTP initiates sporulation. It was found that GTP decrease reduces the rate of ribosomal RNA synthesis but so does the decrease of UTP observed in uracil requiring mutants. Thus the decrease of RNA synthesis may be required for sporulation but it is not sufficient for it. The synthesis of a number of enzymes known to be made during sporulation in other media was examined and it was found that the extracellular and intracellular protease increased only very little after guanine deprivation although a rapid turnover of protein took place. Apparently, another enzyme must be responsible for this turnover. It was also found that a number of nucleases and nucleotidases increase during GTP deprivation, most pronounced being the increase of 5'-mononucleotide producing phosphodiesterase and 5'-nucleotidase. But the same increases were found in a leaky uracil mutant which did not sporulate upon uracil deprivation. The decrease of GTP causes a number of changes in membrane

transport such as a significant decrease in the uptake of uracil, but some other transport changes which had been previously associated with sporulation no longer occurred under these conditions. It is therefore not clear whether any transport changes are necessary for sporulation. The decrease of GTP also caused the accumulation of acetyl-CoA suggesting that fatty acid synthesis may be inhibited. But an examination of fatty acid synthesis by the use of a branched fatty acid mutant did not show any significant inhibition. The reason for the increase of acetyl-CoA will therefore have to be examined. But it is interesting that this increase of acetyl-CoA, which could also be obtained in mutants lacking citrate synthase, caused an increase in succinyl-CoA synthase.

Most interesting was the effect of guanine deprivation on cell wall synthesis. There was a clear cut decrease of wall synthesis after addition of decoyinine, and this phenomenon was not associated with a decrease in the uracil containing cell wall intermediates. Because no guanine containing intermediate or GTP associated reaction in cell wall synthesis is so far known, this phenomenon will have to be further investigated. It is most interesting, because it could be directly associated with the initiation of asymmetric septation.

5. Involvement of branched chain fatty acid synthesis. B. subtilis normally contains about 70% branched chain fatty acids in its membrane phospholipids. These compounds are usually thought of maintaining a high enough membrane fluidity but they could serve a more specific role. To determine whether any particular branched fatty acids was required in large amounts for sporulation or whether the branched fatty acid composition of spores differed from that of vegetative cells, we have analyzed a mutant that requires branched chain fatty acids for growth. We found that any branched chain- or otherwise kinked fatty acid allows normal growth and sporulation of the mutant. No particular branched fatty acid is required in large amounts to allow sporulation. However, when conditions were used under which vegetative cells contained a relatively high frequency of these fatty acids (80%) which still allowed normal growth, the spores produced in the same medium contained a much lower concentration (25%) of straight chain fatty acids. This suggests that the membranes incorporated into the spore select among the fatty acids preferentially those that are branched. Possibly, the round portions of the membrane in the small spore are flexible enough only if the membrane contains a significant fraction of branched fatty acids.

Proposed Course of Project: We want to understand why some antibiotics acting on ribosomes or RNA polymerase can interfere with sporulation while others cannot. This can be studied in general antibiotic resistant mutants, and in temperature-sensitive mutants both of which have been isolated. We also want

to study the effect of thiamine on kasugamycin inhibition and determine whether other antibiotics are similarly effected by thiamine or its analogs. We want to isolate temperature-sensitive and other mutants which can sporulate continuously even in rich media and determine whether GTP binding proteins of these mutants have been altered. We have some indications that part of the sporulation response may be due to reduced methylation by S-adenosyl methionine and want to investigate this phenomenon. We also want to study the effect of a decrease in GTP on cell wall synthesis in the hope that it may explain asymmetric septation and perhaps even contribute to the understanding of regular cell division.

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Boudreaux, D.P., Eisenstadt, E., Iijima, T., and Freese, E.: Biochemical and genetic characterization of an auxotroph of Bacillus subtilis altered in the Acyl-CoA:Acyl-carrier-protein transacylase. Eur. J. Biochem. 115: 175-181, 1981.

Lopez, J.M., Dromerick, A., and Freese, E.: Response of guanosine 5'-triphosphate concentration to nutritional changes and its significance for Bacillus subtilis sporulation. J. Bacteriol. 146: 605-613, 1981.

Uratani-Wong, B., Lopez, J.M., and Freese, E.: Induction of citric acid cycle enzymes during initiation of sporulation by guanine nucleotide deprivation. J. Bacteriol. 146: 337-344, 1981.

Freese, E., Lopez, J.M., and Ochi, K.: Role of guanine nucleotides and of the stringent response to amino acid deprivation in the initiation of bacterial sporulation. In D. Schlessinger (Ed.): Microbiology-1981. Washington, D.C., Amer. Soc. Microbiol., 1981, pp. 11-16.

Freese, E.: Initiation of bacterial sporulation. In H.S. Levinson, A.L. Sonenshein, and D.J. Tipper (Eds.): Sporulation and Germination. Washington, D.C., Amer. Soc. Microbiol., 1981, pp. 1-12.

Lopez, J.M., Ochi, K., and Freese, E.: Initiation of Bacillus subtilis sporulation caused by the stringent response. In H.S. Levinson, A.L. Sonenshein, and D.J. Tipper (Eds.): Sporulation and Germination. Washington, D.C., Amer. Soc. Microbiol., 1981, pp. 128-133.

Lopez, J.M., and Fortnagel, P.: Nitrofurantoin prompts the stringent response in Bacillus subtilis. J. Micro., 1981, in press.

Ochi, K., Kandala, J.C., and Freese, E.: Initiation of Bacillus subtilis sporulation by the stringent response to partial amino acid deprivation. J. Biol. Chem., 1981, in press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  201 NS 01886-11 LMB																					
PERIOD COVERED October 1, 1980 through September 30, 1981																							
TITLE OF PROJECT (80 characters or less) Control of Meiosis and Morphogenesis (Former title, Developmental Cytology)																							
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																							
<table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: E.B. Freese</td> <td style="width: 33%;">Biologist</td> <td style="width: 33%;">LMB NINCDS</td> </tr> <tr> <td colspan="3">OTHERS: M. Chu</td> </tr> <tr> <td></td> <td>Visiting Fellow</td> <td>LMB NINCDS</td> </tr> <tr> <td></td> <td>A. Hartig</td> <td>Visiting Fellow</td> </tr> <tr> <td></td> <td>H. Ishihara</td> <td>Visiting Fellow</td> </tr> <tr> <td></td> <td>Z. Olempska-Beer</td> <td>Visiting Associate</td> </tr> <tr> <td></td> <td></td> <td>LMB NINCDS</td> </tr> </table>			PI: E.B. Freese	Biologist	LMB NINCDS	OTHERS: M. Chu				Visiting Fellow	LMB NINCDS		A. Hartig	Visiting Fellow		H. Ishihara	Visiting Fellow		Z. Olempska-Beer	Visiting Associate			LMB NINCDS
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OTHERS: M. Chu																							
	Visiting Fellow	LMB NINCDS																					
	A. Hartig	Visiting Fellow																					
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	Z. Olempska-Beer	Visiting Associate																					
		LMB NINCDS																					
COOPERATING UNITS (if any) None																							
LAB/BRANCH Laboratory of Molecular Biology																							
SECTION Developmental Biology Section																							
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																							
TOTAL MANYEARS: 4.5	PROFESSIONAL: 3.9	OTHER: 0.6																					
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SUMMARY OF WORK (200 words or less - underline keywords) It was shown that in the yeast <u>Saccharomyces cerevisiae</u> <u>meiosis</u> <u>and sporulation</u> can be initiated not only by <u>nitrogen deprivation</u> <u>but also by partial carbon or phosphate limitation.</u> This is possible only under conditions under which the intracellular concentration can be controlled by the concentration of a compound in the medium. Meiosis and sporulation can also be initiated by the addition of certain <u>inhibitors of nucleotide</u> <u>synthesis</u> and the <u>partial purine deprivation of purine requiring</u> <u>mutants.</u>																							



Project Description:

Objectives: Differentiation of the diploid eukaryote *Saccharomyces cerevisiae* was known to be induced by transfer of gluconeogenically grown cells to potassium acetate. This effect was due to nitrogen limitation. In the attempt to determine which compound is responsible for the control of this differentiation, we wanted to examine whether carbon or phosphate limitation could also lead to sporulation in the presence of all other ingredients needed for normal cell growth. Next, we wanted to determine which carbon, nitrogen, and phosphorus containing compound, was responsible for the initiation. Therefore, we used various inhibitors and mutants to initiate meiosis and as a consequence sporulation,

Methods Employed: At different times, between 20 and 50 hours after cell transfer to the new conditions, asci were counted (400 - 1000 per sample) under the phase contrast microscope. The added carbon sources, e.g. glucose, pyruvate, acetate, and ethanol were measured by enzymatic methods; when needed additional carbon source was added to maintain its concentration within 30% of the original value. ATP was measured by the luciferase assay.

Major Findings:

1. Sporulation of yeast under conditions of carbon, phosphate and nitrogen deprivation. Yeast cells (strain Y55) were grown in a synthetic medium using the same carbon source which was later to be partially deprived. At  $OD_{600} = 1$ , the cells were transferred to the same medium containing different amounts of the carbon source. When the carbon source was acetate or ethanol, the culture continued to grow at the original rate until all carbon was used up. As a consequence, growth stopped completely and no sporulation occurred. In contrast, when pyruvate or dihydroxyacetone were used as carbon source, the cells grew at different rates, depending on the concentration of the carbon compound, and sporulated well at intermediate concentrations while the carbon was still present. Apparently, these carbon sources are taken up only by facilitated transport. The response to galactose was the same as for pyruvate at low galactose concentrations and similar to that of acetate at high galactose concentrations. Apparently, in cells grown at high galactose concentrations a high affinity galactose transport system is repressed, and the cells are not able to induce this transport fast enough to avoid sporulation caused by a sudden reduction in the galactose concentration in the medium. Sporulation could also be initiated by partial limitation of phosphate. In this case, the cells rapidly used up the intracellular phosphate resources and then more slowly the extracellular phosphate on which they could start sporulation. Even with respect to nitrogen deprivation, cells sporulated well

without any nitrogen only when the medium contained acetate. When the medium contained ethanol or pyruvate, some intermediate amounts of slowly metabolizable nitrogen sources such as glycine, phenylalanine, or histidine had to be added. The results have shown clearly that partial deprivation of carbon, nitrogen, or phosphorus compounds can cause meiosis and sporulation of yeast. Presumably, the compound controlling this process is a nucleotide.

2. Effect of inhibitors of nucleotide synthesis. When we tried to inhibit yeast by various base analogs or other compounds, we found that the organism is extraordinarily resistant to most compounds that inhibit bacteria or mammalian cells readily. (This explains why fungal infections generally are very resistant to treatment and diseases caused by these organisms are more difficult to control.) We found that mycophenolic acid, an inhibitor of IMP dehydrogenase, caused some sporulation under special conditions using pyruvate as carbon source. But due to the low solubility of mycophenolic acid, we could not sufficiently increase the concentration of this compound to produce massive sporulation. The same was found for virazole, another inhibitor of IMP dehydrogenase. With decoyinine, which is most effective in bacteria, we observed no inhibition of growth and no sporulation. However, hadacidin, an inhibitor of AMP synthesis, remained soluble up to 150 mM concentration at which it inhibited growth significantly and induced sporulation in more than 10% of the cells (observed after 20 hours). We are encouraged by these findings and want to find other inhibitors or maybe modify chemically the mycophenolic acid to increase its solubility and obtain stronger inhibitory effects.

3. Sporulation in auxotrophic mutants. In order to examine sporulation in auxotrophic mutants, we transferred various auxotrophic markers available from other strains into the genetic background of our strain Y55. We found no sporulation when we limited the concentration of different amino acids in the medium, such as methionine or histidine, or when we limited the concentration of adenine, hypoxanthine, or guanine in a general purine requiring strain; nucleosides were unable to enter yeast cells. Because the mutants were non-leaky, these results do not exclude the possibility that partial limitation of the compound would cause sporulation. We could not control the intracellular concentration of the compounds because we do not yet have the necessary transport mutations and because analogs of pyrimidine or purine bases or of amino acids, which might slowly release the required compound, did not allow any growth of the auxotrophs. However, when we used a specific guanine requiring mutant, which we generated from a haploid strain, we found excellent sporulation under conditions of partial guanine limitation.

Proposed Course of Project: We want to use additional analogs of purine and pyrimidine synthesis and additional mutants, in particular mutants deficient in active transport of the required compound, in order to determine whether the initiation of sporulation is limited to guanine deprivation or can be caused by the specific deprivation of other compounds. We also want to measure the intracellular nucleotide concentration under all conditions under which sporulation has been observed, in order to analyze whether a particular nucleotide decreases (or increases) under all these conditions. The use of amino acid mutants will also allow us to determine whether the stringent response to amino acid deprivation, which in yeast is not related to a ppGpp increase, can initiate sporulation of yeast.

Publications:

Chu, M., Freese, E.B., Hartig, A., and Freese, E. Adaptation of glucose grown *Saccharomyces cerevisiae* to gluconeogenic growth and sporulation. J. Gen. Microbiol., 1981 (in press)

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02364-03 LMB
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Development and Teratology in Rodent Embryo Culture		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: E. Freese, Chief, Laboratory of Molecular Biology LMB NINCDS R.C. Henneberry, Senior Scientist LMB NINCDS  OTHER: A. Bruckner, NIH Expert LMB NINCDS		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Developmental Biology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 1.6	PROFESSIONAL: 1.2	OTHER: .4
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  The major goal of this project is to determine the <u>teratogenic potential</u> of certain <u>lipophilic drugs</u> selected according to their <u>strong growth-inhibition</u> of cultured mammalian cells. New methods permitting the cultivation of <u>early somite-stage embryos</u> have been adopted to avoid the difficulties of absorption, metabolism, and excretion encountered in whole animals. We found that the <u>anticonvulsants valproic acid (VPA)</u> and <u>diphenylhydantoin (DPH)</u> cause <u>developmental defects</u> in a dose-dependent manner. The frequency of defects increased slightly when the developing embryo was examined by more refined methods. A secondary goal was to evaluate the utility of the embryo culture system for <u>developmental biology</u> studies; several pilot experiments were undertaken.		



Project Description:

**Objectives:** The major objective of this project is to evaluate potential teratogens by studying their effects on rodent embryos in culture. The previously inaccessible postimplantation embryo has become available for direct experimentation as a result of recently introduced improved methodology. Rodent embryos can now be explanted at the 2-4 somite-stage and cultivated for up to 48 hours; the embryos develop normally during this period. This approach avoids some of the complications such as absorption, metabolism, and excretion of test compounds encountered in animal studies. The compounds tested were chosen from a list of highly lipophilic substances which are used as drugs and food additives and which have been identified by previous studies of this laboratory as potent inhibitors of growth of mammalian cells in culture.

A second objective is to undertake basic biochemical studies in developmental biology using embryos in culture. Several pilot experiments involving energy metabolism and the appearance of receptors in embryos in culture were performed.

**Methods Employed:** A breeding colony of ICR mice was established and maintained in a twelve hour light - dark cycle and bred on a carefully designed schedule to provide mice containing embryos at the ninth day of gestation. Embryos were dissected, free of both placental material and Reichert's membrane, and cultivated in individual vessels in a medium consisting of 80% rat serum and 20% Tyrode's solution. Compounds to be tested were added directly to the culture medium, and teratogenic effects were assessed by anatomical and histological examinations. Antibiotics were not included in the culture medium to avoid possible complications in the interpretation of results.

Major Findings:

1. Development and refinement of the embryo culture method. Mouse embryos in the ninth day of gestation were explanted from ICR mice and maintained in culture in rat serum for as long as 48 hours during which development proceeds normally; in the absence of test compounds, the incidence of developmental anomalies was very low. When we adopted more refined methods of evaluating of developing embryos, we could detect defects with a greater degree of sensitivity; previously unnoticed anomalies were thus detectable. For example, some neural folds which seemed closed in embryos examined in the stereo microscope were actually open, but in close apposition, when transverse sections were examined by light microscopy. Also asymmetry of the invaginated eye vessel and malrotation of the heart tube were observed by light microscopy at high frequency in embryos treated with suspected teratogens. Electron microscopic (EM) examination detected



additional defects. Ultra thin sections of the hindbrain of drug-treated embryos revealed decreases in numbers of microfilaments and mitochondria as well as larger protrusions in the neuroepithelium. Scanning EM revealed an asymmetry of the neural folds and an irregular surface on the neuroepithelium in drug treated embryos.

2. Teratogenic effects of valproic acid (VPA) and diphenylhydantoin (DPH). We have observed dose-dependent teratogenic effects for VPA and DPH. Both drugs retarded overall growth, reduced the rates of protein and DNA synthesis, and produced several developmental defects including malformations of the heart, brain, spinal cord and craniofacial structures. Such effects were noted at concentrations found in the plasma of patients treated with these drugs. Interestingly, DPH, which is more lipophilic than VPA, has its maximum effect at a concentration about three times lower than that required for VPA.

To compare growth inhibition of mammalian cells in culture and teratogenicity caused by VPA, we measured the growth inhibition of fibroblasts derived from embryonic mice and grown in Eagles medium. The VPA concentration needed to cause 50% growth inhibition of the fibroblast culture was about three times higher than that causing a 50% reduction of growth in cultured embryos. Teratogenic effects were detectable at VPA concentrations eight fold lower than those needed to inhibit fibroblast growth by 50%. Thus, mouse embryo development is extremely sensitive to VPA.

Preliminary experiments on energy metabolism in developing embryos in vitro have been completed in the presence and absence of VPA. Although less glucose was consumed in VPA treated embryos, more lactate per unit glucose consumed accumulated in the VPA treated cultures. This finding may be related to the decrease in number of mitochondria found in VPA treated embryos on EM examination.

3. Feasibility studies to measure the appearance of receptors during embryonic development. We conducted a pilot study to determine whether certain hormone/neurotransmitter receptors can be detected in embryos in culture. For embryos which had developed in utero, specific receptors for dopamine and benzodiazepines were detectable by day 11, increased by day 13, and increased further by day 15. However, the amount of embryonic material needed to detect these receptors was greater than the amount which would be feasible to use in the case of cultured embryos. Other ligands detectable by more sensitive assays will be tested in the future. Also, in situ methods for demonstrating specific hormones/neurotransmitter receptors will be evaluated using sections from embryos at various stages of development.

Proposed Course of Project: The project will continue in its present direction. Additional compounds will be evaluated for teratogenic effects, using the refined system for scoring developmental defects; results of these studies will be correlated with animal studies and clinical findings. Experiments designed to examine the mechanisms by which teratogens cause defects will be attempted when possible. Additional pilot studies will be undertaken in an effort to use the advantages of the embryo culture system in biochemical studies of embryonic development.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02365-03 LMB
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less) Intercellular Communications and Transmembrane Signals		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT PI: Richard C. Henneberry, Senior Scientist LMB NINCDS OTHER: R. Elliott, Visiting Fellow LMB NINCDS		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Molecular Biology		
SECTION Developmental Biology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 2.8	PROFESSIONAL: 1.8	OTHER: 1.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The major goal of this project is to understand the biochemical events involved in the response of individual cells to external signals. We have previously shown that several types of hormone/neurotransmitter receptors increase in several cell lines when the cells are grown in the presence of certain short chain fatty acids. In HeLa cells, e.g., <u>β-adrenergic receptors</u> increase about four-fold in the presence of <u>butyrate</u> , permitting us to analyze the biochemical events in <u>adenylate cyclase</u> activation by hormones and describe a role for <u>phospholipid methylation</u> in receptor function. In FY '81, we showed that a pituitary tumor cell line also contains inducible <u>β-adrenergic receptors</u> and may contain <u>α-adrenergic receptors</u> as well. We continue to explore the mechanisms by which <u>butyrate</u> induces biochemical differentiation; butyrate does not affect the proteins associated with <u>polysomal RNA</u> but does reduce <u>mRNA methylation</u> which may influence translation of these molecules. <u>Studies on benzodiazepine receptors</u> in cultured neurons have continued and the value of this receptor as a neuron-specific marker has been shown.		

Project Description:

**Objectives:** The goal of this project is to better understand the mechanisms by which cell surface receptors for hormones/neurotransmitters convey information across the plasma membrane, and the mechanisms by which the cell responds to these signals. Current objectives are to understand: a) The biochemical events involved in the induction of receptors and other proteins by short-chain fatty acids such as butyrate. b) The interactions between the hormone-receptor complex on the outer surface of the cell and the enzyme, adenylate cyclase, on the inner surface of the plasma membrane. c) The interactions between different receptors on the same cell, especially pairs of receptors such as  $\alpha$ - and  $\beta$ -adrenergic whose occupancy leads to physiologically opposed results.

**Methods Employed:** Standard binding assays with radioactive ligands were used to quantify receptor numbers on whole cells and in membrane preparations. Cyclic AMP accumulation in whole cells or adenylate cyclase activity in cell-free extracts depends on quantitation by radioimmunoassay of the cyclic AMP produced per unit time. Cellular RNA was radioactively labeled (when required) by growing cells in the presence of radioactive precursors. [ $^{14}\text{C}$ ]-uridine was used to label RNA per se and [ $^3\text{H}$ ]-methyl-methionine was used to measure nucleic acid methylation. Sodium formate was routinely included when labeling with tritiated methyl-methionine to prevent incorporation of methyl groups into purine rings. Total cytoplasmic RNA was extracted from post-mitochondrial supernatant fluids from cultured cells or tissues by standard methods and poly(A)-containing RNA isolated by oligo(dT)-cellulose chromatography. The various size classes of mRNA were determined by polyacrylamide gel electrophoresis in the presence of SDS or formamide. Highly purified mRNA from cultured cells and from newborn rat brain was translated in vitro in a reticulocyte lysate system.

Major Findings:

1. Inducible receptors in GH<sub>3</sub> cells. We found that the rat pituitary tumor cell line, GH<sub>3</sub>, which produces both growth hormone and prolactin contains receptors for  $\beta$ -hydroxylated catecholamines. The number of  $\beta$ -adrenergic receptors is increased when the cells are exposed to butyrate over a time course of 12 hours, similar to the induction seen with HeLa cells. We also found a previously unreported temperature-dependent degradation of these receptors during the binding assay which may have implications for receptor binding studies in general. Preliminary pharmacological evidence indicates that the GH<sub>3</sub> cells may contain  $\alpha$ -adrenergic receptors in addition to the  $\beta$ -adrenergic receptors.



2. Mechanism of induction by butyrate. The mechanism through which butyrate induces its many biochemical changes in different cell types is unknown. In HeLa cells butyrate must be metabolized before it can function as an inducer, but subsequent steps in the induction process are not known. New proteins appear in the cell as a result of exposure to butyrate but it is not known whether the regulation of synthesis of these proteins is at the level of transcription or translation. This problem has been approached in several ways during this reporting period: (1) Capping and methylation of mRNA are recognized as important mechanisms for regulating translation of mRNA. In HeLa cells butyrate was found to inhibit mRNA methylation, with possible implications in the regulation of translation. Current efforts are directed toward establishing whether this change in methylation occurs only in the cap and whether the mRNAs from untreated and butyrate treated cells are differentially translated in an in vitro system. (2) A regulatory role for the proteins associated with polysomal mRNA has often been proposed. The proteins associated with polysomal RNA were isolated from untreated and butyrate-treated HeLa cells and analyzed by polyacrylamide gel electrophoresis. In both cases a single protein with a molecular weight of 74,000 was found, implying that butyrate does not function through alterations in this potential regulatory mechanism. Others have reported that butyrate inhibits DNA synthesis and histone deacetylation resulting in accumulation of particular histones; this effect has been invoked by some investigators as a possible explanation of butyrate's ability to induce numerous biochemical changes. However, in a mutant HeLa cell line selected for its ability to grow normally in the presence of butyrate, we have shown that  $\beta$ -adrenergic receptors are induced indicating that inhibition of DNA synthesis is not required for induction. We are currently examining the effects of butyrate on histone acetylation in this mutant.

3. Benzodiazepine receptors. Receptors for the benzodiazepines, such as valium, occur in both the CNS and in peripheral tissues and can be shown to be of two distinct types. We have previously shown that only neurons contain CNS type benzodiazepine receptors and have used selective tissue culture methods to influence the relative numbers of different cell types in primary cultures from embryonic rat brain. The value of the benzodiazepine receptor as a specific marker for identification of neurons in culture has been shown. Comparisons of this marker with other, established neuron-specific markers are in progress.

Proposed Course of Project: The project will continue in its present direction with the goal of understanding the molecular mechanisms underlying the receipt of biochemical signals by an individual cell and the response of the cell to



those signals. Emphasis will be placed on the induction of receptors by butyrate, since an understanding of that phenomenon should contribute to the understanding of regulation of receptor synthesis in general. Analysis of the components of the receptor-adenylate cyclase complex will also continue. Studies on the catecholamine receptors in GH<sub>3</sub> cells will emphasize the relationship between the  $\alpha$ - and  $\beta$ -adrenergic receptors and the response of the cell to these physiologically opposed signals. The suitability of the benzodiazepine receptor as a neuron-specific marker will also be assessed in a comparative study with other generally accepted markers.

#### Publications:

Parent, B., Tallman, J.F., Henneberry, R.C., and Fishman, P.H.: Appearance of beta-adrenergic receptors and catecholamine-responsive adenylate cyclase activity during fusion of avian embryonic muscle cells. J. Biol. Chem. 255: 7782-7786, 1980.

Fishman, P.H. and Henneberry, R.C.: Induction of ganglioside biosynthesis in cultured cells by butyric acid. In Sweeley, C.C. (Ed.): Cell Surface Glycolipids. New York, Academic Press, 1980, pp. 223-240.

Gallager, D.W., Mallorga, P., Ortel, W., Henneberry, R.C., and Tallman, J.F.: [<sup>3</sup>H]-diazepam binding in mammalian central nervous system: A pharmacological characterization. J. Neuro. Sci. 1:218-225, 1981.





# ANNUAL REPORT

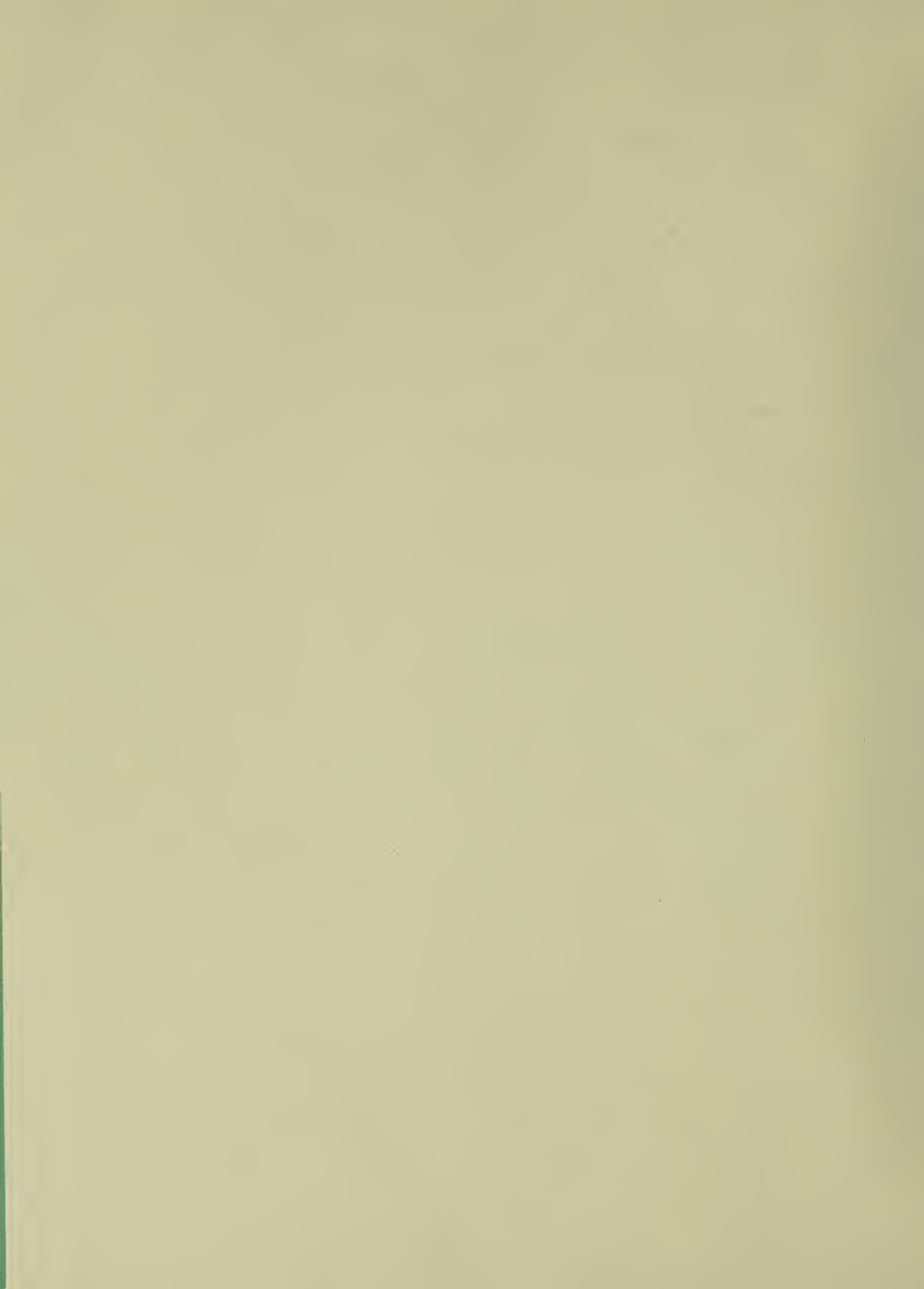
October 1, 1980 through September 30, 1981

## Laboratory of Neuro-otolaryngology

National Institute of Neurological and Communicative Disorders and Stroke

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## ANNUAL REPORT

October 1, 1980 through September 30, 1981  
Laboratory of Neuro-otolaryngology, IRP  
National Institute of Neurological and  
Communicative Disorders and Stroke

Jürgen Fex, M.D., Ph.D., Chief

The Laboratory has continued its multidisciplinary approach with the focus on the inner ear and cochlear nucleus of mammalian species, of normal animals as well as of genetically deaf animals. The two Projects of the Laboratory have been advanced, these being Project Number Z01 NS 02216 06 LNO, Inner Ear Neuronal Mechanisms: A Multidisciplinary Analysis, respectively Project Number Z01 NS 02217 06 LNO, Synaptic Transmission and Neuronal Connections of the Mammalian Cochlear Nucleus. In particular, during this fiscal year, the Laboratory contributed with the following new knowledge.

Immunocytochemical and microdissection techniques worked out in the Laboratory have been applied to the study of the distribution of small peptides and proteins in the cochlea of small mammals, including the mouse, rat, guinea pig and cat. A paper on the enkephalin-like immunoreactivity of olivocochlear nerve fibers in cochlea of guinea pig and cat has been published. These results have given the impetus for studies designed to determine the specific nature of the enkephalin-like substance in the cochlea, using high-performance liquid chromatography (HPLC) and radio immuno assays (RIA). Studies are also underway to determine the nature of the receptor(s) that corresponds to the enkephalin-like substance in the cochlea.

Immunocytochemical techniques have provided indication that two enzymes, aspartate aminotransferase (AAT) and glutaminase, associated with the metabolism of the excitatory amino acids glutamate and aspartate, are present in relatively high concentrations in the cochlea and in the auditory nerve, as shown at the light microscopy level. Experiments are underway to indicate the exact localization of these enzymes in the organ of Corti. Both these enzymes have also been shown to be present at relatively high concentrations in auditory nerve endings in the cochlear nucleus, adding to our previous evidence that glutamate and/or aspartate may act as neurotransmitter(s) of the auditory nerve. A manuscript on the presence of AAT in auditory nerve endings as studied at the electron microscope level has been submitted for publication.

Immunocytochemical techniques have also been applied to determine enkephalin-, AAT- and glutaminase-like immunoreactivity in other parts of the central nervous system. In particular, the search for AAT- and glutaminase-like immunoreactivity is being carried out in such regions of the central nervous system in which glutamate or aspartate are likely neurotransmitter candidates. Our aim is to determine whether one or both of these enzymes may serve as a marker for glutamergic or aspartergic neurons.

Also, we have extended our demonstration of enkephalin-like immunoreactivity of olivocochlear neurons in the cochlea to a study of cells of origin of such neurons, in the superior olivary complex of the guinea pig. We have shown that a group of such cells show both enkephalin-like immunoreactivity and acetylcholinesterase staining. Our results are in manuscript form and will be submitted for publication shortly.

A series of findings on the morphology of the cochlear nucleus of the mouse that were mentioned in last year's Annual Report has now been published under the descriptive titles "Cell types in the cochlear nucleus of the mouse and their birth dates as determined by autoradiography", "The cochlear nucleus of the Reeler mutant mouse", and "Acetylcholinesterase-positive efferents in the cochlear nucleus of the mouse."

The synapse of the auditory nerve with cells in the cochlear nucleus has been subject to morphological studies. A technological innovation using deep-etched, unfixed slices of guinea pig anteroventral cochlear nucleus permitted high resolution examination of components of the cytoskeleton in the postsynaptic complex. The findings led to the postulation that a fine meshwork of filaments anchor receptors in the membrane to a lattice of adjacent microfilaments, perhaps limiting the mobility of receptors and helping to maintain the curvature of the postsynaptic membrane. This study has been accepted for publication.

The maturation of primary auditory nerve terminals in the rostral anteroventral cochlear nucleus has been studied in serial thin sections and freeze-fracture preparations under the electron microscope, in the rat. Also, species differences in the presynaptic membrane of these terminals have been observed. Manuscripts on these studies will be submitted for publication shortly.

Our studies of the physiology and pharmacology of synaptic transmission in the cochlear nucleus have brought additional evidence that the neurotransmitter of the auditory nerve is an excitatory amino acid. Thus, we have shown that Baclofen, a commonly used antispastic drug, which is believed to block the release of excitatory amino acid neurotransmitters, suppresses or blocks early peaks in the series of brainstem auditory evoked potentials. This likely corresponds to the auditory distortion which clinically is one side effect of this drug.

Synaptic activity of the auditory nerve in the anteroventral cochlear nucleus has been studied using electrophoretic microtechniques as described in previous Annual Reports. Responses to bicuculline, strychnine and depressant amino acids have been investigated. Findings indicate that such amino acids are not involved in the response to tone bursts at the best tonal frequency for eliciting responses or in the suppression of such responses by a second, higher frequency tone. A report on this has been submitted for publication.

The activity of single cells receiving auditory nerve synapses in the anteroventral cochlear nucleus has been studied in terms of excitatory and inhibitory interactions between two tonal stimuli presented simultaneously to the same ear. New is the finding of a lateral inhibition of activity in these cells, most likely separate from the inhibition-like response, the much studied two-tone suppression that originates in the cochlea. The two phenomena may be functionally overlapping.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> <b>INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 NS 02216 06 LNO
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less) Inner Ear Neuronal Mechanisms: A Multidisciplinary Analysis		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	J. Fex R. A. Altschuler R. L. Gulley D. W. Hoffman M. R. Martin R. J. Wenthold	Chief, LNO Staff Fellow Senior Staff Fellow Staff Fellow Senior Staff Fellow Senior Staff Fellow LNO NINCDS LNO NINCDS LNO NINCDS LNO NINCDS LNO NINCDS LNO NINCDS
OTHER:           None		
COOPERATING UNITS (if any) None		
LAB/BRANCH Laboratory of Neuro-otolaryngology		
SECTION		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
5.5	2.7	2.8
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<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The long-range purpose of the project is to study the biochemistry, morphology, pharmacology and physiology of inner ear neurons and other cells and to describe the mechanisms of their interactions. 1. <u>Enkephalin-like immunoreactivity</u> using the <u>PAP-technique</u> has been demonstrated in efferent synapses on outer hair cells in the guinea pig cochlea at the ultrastructural level. Biochemical studies to identify the opiate(s) in the cochlea are in progress, using high-performance liquid chromatography (HPLC) and radio immunoassay (RIA) techniques. Biochemical opiate receptor studies on the cochlea are in progress. 2. Cells of origin of <u>olivocochlear neurons</u> have been shown to both contain <u>acetylcholinesterase</u> and to exhibit <u>enkephalin-like immunoreactivity</u> . 3. <u>Aspartate aminotransferase (AAT)</u> - and <u>glutaminase-like immunoreactivity</u> at a high level has been found in <u>spiral ganglion cells</u> and at <u>synapses</u> in the organ of Corti.		

## Project Description:

**Objectives:** To study the biochemistry, morphology, pharmacology and physiology of inner ear neurons and other cells and their interactions and to describe the mechanisms of these interactions.

The following subprojects are now serving these objectives:

I. An immunocytochemical study of the distribution of small peptides and proteins in the cochlea of mammals.

- a. Methionine enkephalin
- b. Aspartate aminotransferase (AAT)
- c. Glutaminase

II. A histochemical/immunocytochemical study of cells of origin of efferent olivocochlear neurons in the cochlea showing enkephalin-like immunoreactivity.

III. A biochemical study to identify the enkephalin-like immunoreactivity in the cochlea.

IV. A biochemical study to determine the receptor(s) that may correspond to the presence of methionine enkephalin-like immunoreactivity in the cochlea.

Methods Employed:

I. a. Antibodies to methionine enkephalin were generated in rabbits using methionine enkephalin coupled with glutaraldehyde to bovine thyroglobulin. Mice, rats, guinea pigs and cats were anesthetized and transcardially perfused with fixation fluid. The cochleae were removed and locally perfused with the same fixation fluid, rinsed and prepared for surface preparations of the organ of Corti or for cryostat sections of the cochlea. Modifications of the indirect fluorescence technique of Coons were used on both preparations. After incubation with the antiserum against methionine enkephalin, FITC-labeled swine anti-rabbit immunoglobulin antiserum was used for the second incubation. The final preparations were viewed with a Zeiss photomicroscope under epi-fluorescent illumination. Routine specificity studies were carried out.

Immunoelectron microscopic techniques were developed for ultrastructural localization of enkephalin-like immunoreactivity at outer hair cell synapses in the organ of Corti of the guinea pig. The peroxidase-anti-peroxidase (PAP) technique was used on surface preparations of the organ of Corti. Based on the findings from thick section under the light microscope, segments were selected, mounted in blocks and thin sectioned for electron microscopy.

b. Antibodies against AAT were generated in rabbits and the antisera characterized. These were used as the antisera for methionine enkephalin as described above, under (a), for studies at the light microscopy level.

c. Antiserum to glutaminase was obtained from Dr. Norman Curthoys (University of Pittsburgh, Pittsburgh, PA) and used as described above for



methionine enkephalin, under (a), for studies at the light microscopy level.

II. Retrograde transport studies were done using horseradish peroxidase or wheat germ agglutinin as the tracer substances. Perilymph was withdrawn through the oval window of the left cochlea and replaced with 10  $\mu$ l of tracer substance. For horseradish peroxidase localization serial sections were cut through the brainstem with a vibrating microtome and processed according to the method of Mesulam using tetramethyl benzidine as substrate. Reacted sections were rendered stable in methyl salicylate according to methods developed in this laboratory by J. C. Adams. Animals injected with wheat germ agglutinin had 10  $\mu$ m cryostat sections cut through the brainstem and indirect immunofluorescence techniques applied using antiserum to wheat germ agglutinin. Enkephalin-like immunoreactive staining was determined on guinea pigs with and without wheat germ agglutinating injections. The same protocol used for wheat germ agglutinin immunoreactivity was followed. In the animals with wheat germ agglutinin injections, enkephalin and wheat germ agglutinin antibodies were applied on adjacent sections.

The technique developed by Lundberg et al to demonstrate vasoactive intestinal polypeptide immunoreactivity and acetylcholinesterase staining in the same cells was applied with minor changes in this study, to demonstrate enkephalin-like immunoreactivity and acetylcholinesterase in the same cells in the same sections.

III. Female NIH strain guinea pigs were anesthetized with chloral hydrate i.p. (1 ml/kg 17.5% solution), decapitated, and the cochlea removed into 0.05% trifluoroacetic acid in 22% acetonitrile. Two cochleae were immediately sonicated in 1 ml in a Sonifier cell disruptor. The sonicate was centrifuged for 2 minutes in an Eppendorf microfuge in a cold room. The supernatant was injected onto a Waters microBondapak octadecylsilica column, 10  $\mu$ m particle diameter. Substances of interest were eluted isocratically with the above buffer pumped at 0.5 ml/min by a Waters M6000A pump and monitored with a Schoeffel 770 variable wavelength detector set at 206 nm wavelength and 0.01 absorbance units full scale. The limit of sensitivity of the optical detection of small peptides in this system approaches 1 ng. Fractions were collected and frozen in liquid nitrogen. Individual fractions were lyophilized overnight and reconstituted in radio immuno assay (RIA) buffer for assay. Both solvent and acid are volatile and did not interfere with the RIA.

RIAs for methionine enkephalin were performed with kits from Immunonuclear Co., or with antisera raised in our laboratory. The procedure in either case is a competitive binding assay, involving an incubation at 4°C for 16-22 hr. in the presence of 125-250 pg/ml <sup>125</sup>I-methionine enkephalin. Bound <sup>125</sup>I-methionine enkephalin was precipitated with saturated ammonium sulfate in the presence of carrier rabbit immunoglobulin after incubation and counted in a gamma counter. Quantitation was in relation to a standard curve of percent bound versus added synthetic methionine enkephalin.

IV. Opiate receptor binding was assayed using a glass fiber filter technique. Whole cochlea were removed from female NIH strain guinea pigs (150-250 g), decapitated under anesthesia with 1 ml/kg 17.5% chloral hydrate i.p. Four cochlea from two animals were pooled in 2.2 ml of buffer composed of 50 mM Tris HCl, 100 mM NaCl and 1% BSA, pH 7.6 at 4°C, and sonicated with a Sonifier cell dis-



ruptor. Ten  $\mu\text{l}$  of dextrorphan tartrate or levorphanol tartrate (to a final concentration of 10  $\mu\text{M}$ ) or 10  $\mu\text{l}$  of buffer, were added to 170  $\mu\text{l}$  of sonicate. Twenty  $\mu\text{l}$  of  $^3\text{H}$ -naloxone (New England Nuclear, 50  $\mu\text{Ci}/\text{mmol}$ ) in buffer were added to provide final concentrations of from 1 to 20 nM  $^3\text{H}$ -naloxone. Tubes were then incubated at 4°C in the dark for 1 hr. Tubes were decanted onto Whatman GF/B filters in a Millipore filter holder, and each tube rinsed with 5 ml ice-cold buffer onto the filter. Vacuum was applied until the rinse was drawn through the filter and then released. A further 5 ml wash as above was applied and drawn through by vacuum. Filters were vortexed vigorously in a scintillation vial containing 1 ml 0.1% Triton X-100 until a slurry formed, after which 10 ml Aquasol-2 was added to the vial and briefly vortexed before counting by liquid scintillation spectrometry.

### Major Findings:

I. a. The major findings at the light microscopy level were described in the previous year's Annual Report. The data concerning cat and the guinea pig were the same and have now been published. Shortly, the findings led to the conclusion that efferent, olivocochlear neurons of the cochlea, synapsing predominantly with primary auditory nerve fibers from the inner sensory cells or with these sensory cells, contain enkephalin-like immunoreactivity. Also, the findings indicate that endings of olivocochlear neurons that synapse predominantly with outer hair cells contain enkephalin-like immunoreactivity. It has been confirmed at the ultrastructural level that these latter synapses on outer hair cells contain enkephalin-like immunoreactivity.

b. AAT-like immunoreactivity in the organ of Corti has been localized to synaptic regions of outer hair cells. In the modiolus of the cochlea, spiral ganglion cells show relatively strong AAT-like immunoreactivity.

c. Glutaminase-like immunoreactivity in the organ of Corti has been localized to synaptic regions of outer hair cells. At the inner hair cells, the findings are less clear and studies on this point are in progress. In the modiolus, spiral ganglion cells show relatively strong immunoreactivity.

II. Cells of origin of olivocochlear neurons in the cochlea were localized in the body and hilus of the lateral superior olive in the brainstem of the guinea pig. Such cells were shown to contain acetylcholinesterase and enkephalin-like immunoreactivity.

III. Preliminary results agree with the findings reported above under I.a., that there is enkephalin-like immunoreactivity in the cochlea.

IV. Preliminary results indicate there are opiate receptors in the cochlea.

### Significance to Biomedical Research and the Program of the Institute:

This multidisciplinary study on inner ear structures and mechanisms, including the sensory cells and the neurons and their interactions, provides new knowledge on the poorly understood mechanisms of hearing. Such knowledge will lead to better understanding of the causes of sensory deafness and nerve deafness and will most likely lead to better management of hearing disorders. The study

is of direct significance to biomedical research also in a more general sense because it provides new knowledge on neurotransmitters and/or related enzymes with important functions in the central nervous system, not only in auditory regions.

In particular, referring to subprojects: I a. together with II, III and IV. The finding that cells of origin of olivocochlear neurons in the mammalian organ of Corti contain both acetylcholinesterase and enkephalin-like immunoreactivity adds to previous evidence that such neurons may release both acetylcholine and an opiate when activated. Our evidence that acetylcholine-opiate neurons may exist is stronger than any other evidence at present available. To our knowledge we are the first to study histochemically the distribution of immunoreactivity attributable to the important, neurotransmitter-related enzymes cytoplasmic AAT and glutaminase. The findings of both AAT- and glutaminase-like immunoreactivity in spiral ganglion cells indicates that these enzymes may be used as markers for glutamergic and aspartergic neurons.

#### Proposed Course:

We are continuing our study of enkephalin-like immunoreactivity and opiate receptors in the hearing organ of mammals. We expect to confirm our preliminary finding that such receptors are present and then to be able to determine the nature and the distribution in the hearing organ of such receptors and to follow up the studies with pharmacological experiments.

We will determine the exact localization of AAT- and glutaminase-like immunoreactivity in the hearing organ and, depending on the findings, try to determine the functional implication of the presence in the hearing organ of these enzymes at relatively high concentrations.

#### Publications:

Fex, J. and Altschuler, R. A.: Enkephalin-like immunoreactivity of olivocochlear nerve fibers in cochlea of guinea pig and cat. Proc. Nat. Acad. Sci. USA 78: 1255-1259, 1981.

Fex, J. and Martin, M. R.: Lack of effect of DL- $\alpha$ -amino adipate, an excitatory amino acid antagonist, on cat auditory nerve responses to sound. Neuropharmacology 19: 809-811, 1980.

Wentholt, R. J. and McGarvey, M. L.: Analysis of proteins in the stria vascularis of the normal and the waltzing guinea pig. Acta Oto-Laryngologica 90: 66-73, 1980.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02217 06 LNO
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less) Synaptic Transmission and Neuronal Connections of the Mammalian Cochlear Nucleus		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	J. Fex R. A. Altschuler J. W. Dickson R. L. Gulley M. R. Martin R. J. Wenthold	Chief, LNO Staff Fellow Senior Staff Fellow Senior Staff Fellow Senior Staff Fellow Senior Staff Fellow  Chief, FNS
		LNO NINCDS LNO NINCDS LNO NINCDS LNO NINCDS LNO NINCDS LNO NINCDS  LNNS NINCDS
OTHER:	T. S. Reese	
COOPERATING UNITS (if any) D. E. Mattox, Univ. of Texas, Health Science Center at San Antonio, Dept. of Surgery, San Antonio, Texas 78284; C. Rickets, University College, Dept. of Physiology, London, England		
LAB/BRANCH Laboratory of Neuro-otolaryngology		
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<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The purpose of the project is to study the biochemistry, morphology, pharmacology and physiology of synaptic transmission and neuronal connections of nerve cells of the mammalian cochlear nucleus. New results have been added to previous findings of the Laboratory to give increasing support for a <u>neurotransmitter role for glutamate/aspartate in the cochlear nucleus</u> : 1. Corresponding to terminals of the auditory nerve, rings of <u>aspartate aminotransferase(AAT)- and glutaminase-like immunoreactivity</u> have been found around cells in the <u>ventral cochlear nuclei</u> . 2. <u>Baclofen</u> , which is believed to block release of excitatory amino acids, strongly <u>suppresses</u> peaks 2, 4 and 5 of the <u>brainstem auditory evoked potential</u> of cat. - New immunocytochemical findings on the distribution of <u>AAT and glutaminase in retina, hippocampus and cerebellum</u> , together with our new findings on the <u>cochlear nucleus and cochlea</u> , indicate that the two enzymes may serve as <u>markers for glutamergic/aspartergic neurons</u> . Studies of the <u>cochlear nucleus, on development in normal mouse, and on structure in normal and reeler mouse</u> , have been published. An important technical innovation has been adapted to a study of <u>postsynaptic densities examined in deep-etched, unfixed slices of guinea pig anteroventral cochlear nucleus</u> .		

Project Description:

Objectives: To study the biochemistry, morphology, pharmacology and physiology of synaptic transmission and neuronal connections of nerve cells in the mammalian cochlear nucleus.

The following subprojects are now serving these objectives:

I. Synaptic transmission. Morphology.

- a. Immunocytochemical localization of aspartate aminotransferase (AAT)-like immunoreactivity in the guinea pig cochlear nucleus and other CNS regions at the light microscopy and electron microscopy level.
- b. Immunocytochemical localization of glutaminase-like immunoreactivity in the guinea pig cochlear nucleus and other CNS regions at the light microscopy level.
- c. An electron microscopy study on the organization of the post-synaptic complex.
- d. Electron microscopy studies of synapse maturation.

II. Synaptic transmission. Physiology/pharmacology.

- a. A microiontophoretic study of bicuculline, strychnine and depressant amino acid responses in the anteroventral cochlear nucleus of the cat.
- b. A microiontophoretic study of lateral inhibition in the anteroventral cochlear nucleus of the cat.
- c. A study of effects of baclofen on the brainstem auditory evoked potential of the cat.

III. Axonal transport.

IV. Structural and developmental studies of the cochlear nucleus of the mouse.

- a. Morphology of the cochlear nucleus of the normal and reeler mutant mouse.
- b. Histogenesis of the cochlear nucleus of the mouse.
- c. Acetylcholine-esterase-positive fibers and cell bodies in the cochlear nuclei of normal and reeler mutant mice.

Methods Employed:

I. a. The methods employed were partly described in last year's Annual Report and were shortly as follows: Antibody to AAT was made using the commercially available cytoplasmic form of the enzyme obtained from pig heart. This preparation was purified once on a Sephadex G 150 column. Fractions containing AAT activity were pooled and used for the production of antibodies in rabbits. Antibodies obtained following this method reacted with both pig heart AAT and guinea pig brain AAT. To insure that antibodies were specific for AAT, our efforts were directed at obtaining a homogenous preparation of AAT. The only satisfactory method was found to be extraction of AAT from SDS polyacrylamide



gels. This extracted preparation cross-reacts with our antibody. This purified preparation was used for the preparation of antibodies in rabbits. These different antisera to AAT were used in immunocytochemical studies on the guinea pig cochlear nucleus, retina, hippocampus and cerebellum. Indirect immunofluorescence techniques were used on cryostat sections for light microscopy visualization. Immunoelectronmicroscopy techniques were developed for ultrastructural localization of AAT-like immunoreactivity, particularly in the rostral anteroventral cochlear nucleus, using the peroxidase-anti-peroxidase (PAP) technique on vibratome sections of aldehyde fixed brains.

b. Glutaminase for control experiments and antiserum to glutaminase were obtained from Dr. Norman Curthoys (University of Pittsburgh, Pittsburgh, PA). Immunofluorescent techniques, similar to those described above for AAT, were used on cryostat sections of the guinea pig cochlear nucleus, retina, hippocampus and cerebellum.

c. This study adapted an important technical innovation which is described in a corresponding paper already accepted for publication. Very briefly, post-synaptic densities were examined in deep-etched, unfixed slices of guinea pig anteroventral cochlear nucleus.

d. Primary auditory terminals in the rostral anteroventral cochlear nucleus were examined in conventional serial thin section and in freeze-fracture preparation under the electron microscope.

II. a. and b. The microiontophoretic and surgical methods used were essentially the same as those listed in previous Annual Reports and were shortly as follows: Experiments were performed in a sound chamber on anesthetized cats. Action potentials of single units were recorded extracellularly from the anterior and posterior divisions of the anteroventral cochlear nucleus using the 4 M NaCl-filled center barrel of seven barrel microelectrodes. Five of the six outer barrels contained the compounds to be ejected electrophoretically using standard procedures as described by Curtis, 1964. In the brainstem auditory system, those cells that receive large calyceal endings show a peculiar waveform associated with unit action potentials. Preceding each action potential by approximately 0.5 msec is a "prepotential" that has been shown to be presynaptic. The presence or absence of prepotentials were used for categorizing unit types. The rate of unit discharge was computed and displayed on a potentiometric chart recorder for on-line assessment of drug action. Averages and histograms were made on-line with a PDP-11 computer, records were kept on magnetic analog tape.

For II a., the lateral inhibition study, the sound stimulation took place as follows: Tone burst durations of 60 msec duration at the characteristic frequency (CF) were used and a control poststimulus-time histogram constructed from 250 or 500 consecutive tone bursts. To observe the effect of side band tones on acoustically driven units, a 20 msec suppressing tone which started 20 msec after the onset of the CF (exciting) tone was added. Frequencies higher than CF were used in all cases. The effects of side band tones both on the responses to CF tone bursts and on the amino acid-induced excitation were determined by constructing poststimulus-time histograms from 250 or 500 consecutive tone bursts. Once a non- or low- (less than 15 Hz) spontaneously active non-prepotential (NPP) unit had been isolated, its characteristic frequency (CF) and threshold were



measured. The response pattern to 30 msec tone bursts were determined from post-stimulus-time histograms using CF tones 6 to 20 dB above threshold.

C. Experiments were performed in a sound proofed room and 7 adult (1.9-3.1 kg) male and female cats were anaesthetized with 40 mg/kg pentobarbital; anaesthesia was maintained with a 5 mg/ml solution of pentobarbital given via a catheter in the brachial vein either by periodic injection or constant infusion. Body temperature was maintained between 36 and 38°C with a thermostatically controlled heating pad. The external ear was removed, the tympanic bulla opened and its septum removed to expose the cochlea. Acoustic stimuli were presented in a closed sound system through a 1 in. condensor microphone. A wire electrode was placed adjacent to the round window (reference electrode on the tongue) to assess the threshold for click-evoked electrical activity.

For recording the brainstem auditory evoked potential (BAEP), a stainless steel screw was fixed at the vertex and a reference wire electrode placed in the lower caudal quadrant of the portion of the temporal bone forming the external meatus near the tympanic membrane. Clicks (0.1 msec, 1.0 V) were presented at 10 per second. Signals were amplified by a Grass P511 amplifier. The bandpass was 10 Hz to 10 KHz with a gain of 50,000. Responses to 600 consecutive stimuli were averaged by a PDP 11/40 computer. Responses to click stimuli 0, 5, 10, 20 and 40 dB above the threshold for the compound action potential measured at the round window were computed. Two to three control responses for each stimulus intensity were computed before baclofen, dissolved in saline, was given intravenously. Baclofen was given as a single dose by slow infusion over a 6 to 8 minute period. Doses of 2 and 3 mg/kg were given to 3 animals, respectively, for a total of 6 animals. GABA (3 mg/kg) in saline, was given to one animal as a control. BAEP responses to each stimulus intensity were computed at 15 minute intervals for the first hour and then at 90, 120 and 240 (and occasionally at 360) minutes after drug injection.

III. Female guinea pigs, 250-325 g, were used in all studies. Optic nerve proteins were labeled by injecting 500  $\mu$ Ci  $^{35}$ S-methionine in 5  $\mu$ l PBS into the vitreous body of each eye. The animal was killed after 6 h and the superior colliculi were removed. The post mitochondrial fraction was obtained and either stored frozen or extracted immediately. Several different detergents and conditions were tried. Final data were obtained using 1% deoxycholate (DOC) or 1% Triton X100 (TX) at pH 7.5 or 8.1. Extractions were carried out at 22°C for 30 min. Extracts were spun 100,000 g, 30 min and pellets were washed with H<sub>2</sub>O. Pellets were solubilized for SDS gel electrophoresis.

Post-synaptic densities (PSD) were obtained using the method of Siekewitz.

IV. a. Cell types in the cochlear nucleus of the mouse were differentiated using a cresyl violet/luxol fast blue stain on 10  $\mu$ m thick paraffin embedded sections which had been fixed in Bodian's fixative. Cell types were characterized by shape and size of the cell body, distribution and appearance of the Nissl substance, the size, shape and location of the nuclei, and the location within the nucleus.

Material from Reeler mutant mice, and their normal littermate, as controls, were prepared and cell types differentiated using the procedures and criteria

described above. The distribution and number of granule cells in the cochlear nucleus of normal and Reeler mutant mice were estimated using a computer based system interfaced with a microscope stage. The program stored data concerning the total number of granule cells within any  $130 \mu\text{m}^2$  area of a section through the cochlear nucleus. This program gives information about differences in estimated total number of granule cells, their density and distribution. The program is adaptable to any cell type in the cochlear nucleus that one might chose to quantify in these terms.

b. Cell birth dates were determined using tritiated thymidine autoradiography. Animals were injected with the labeled compound,  $5 \mu\text{Ci/g}$  body weight intraperitoneally, at half day intervals between gestation days 5 and 19.5 and on postnatal days 2, 4, 6, 8, 10, 12 and 14. Fetuses of gestation day 8.5, 9.5 and 10.5 were injected using a laparotomy procedure. Animals were killed at 21 days postnatal and fixed in iced Carnoy's solution, paraffin embedded and cut as  $6 \mu\text{m}$  thick sections. Mounted sections were dipped in K2 emulsion ( $3 \mu\text{m}$  thick) and developed after 6 weeks in D19B. Sections were then stained with Erlich's hematoxylin.

c. Cholinesterase activity in the cochlear nucleus of the mouse was demonstrated using the direct coloring method of Karnovsky and Roots. Animals were fixed in 20% formalin in isotonic sodium sulphate at  $37^\circ\text{C}$ . Brains were stored in 10% formalin in isotonic sodium sulfate at  $4^\circ\text{C}$  for 6-18 hours before being transferred to a 20% alcohol solution, also at  $4^\circ\text{C}$ . The tissue was stored for an additional 6-24 hours and then frozen sections were cut at  $40 \mu\text{m}$ . Sections were incubated in the Karnovsky and Roots solution for 90 minutes at  $37^\circ\text{C}$ . The sections were then mounted and sometimes counterstained with cresyl violet. In control experiments BW 284C 51 was used in the incubate to inhibit true acetylcholinesterase and ethopropazine HCl to inhibit pseudo-cholinesterases.

### Major Findings:

I. a. Immunofluorescence techniques showed rings of AAT-like immunoreactivity around cells in the ventral cochlear nuclei. These correspond to auditory nerve terminals as previously demonstrated in this Laboratory by anterograde transport autoradiography. After eighth nerve lesions such rings were no longer seen in the ipsilateral cochlear nucleus. Instead only a few small patches of immunofluorescence could be seen around the cells. At the ultra-structural level in the rostral AVCN we demonstrated AAT-like immunoreactivity on bushy cells in terminals containing large round vesicles and exhibiting other morphological characteristics of auditory nerve terminals.

AAT-like immunoreactivity at relatively high concentrations was also seen in other CNS neurons that may use glutamate or aspartate as neurotransmitter, in retina, hippocampus and cerebellum.

The findings on AAT in the cochlear nucleus have been submitted for publication. The findings on AAT in other parts of the CNS are being prepared for publication.

b. As for AAT, glutaminase-like immunoreactivity has been localized to synaptic regions of the auditory nerve in the cochlear nucleus and to other

structures of the CNS that may use glutamate or aspartate as neurotransmitter.

The findings are being prepared for publication.

c. The postsynaptic density seen in thin sections corresponds to a meshwork of 4 nm filaments associated with intramembrane particles at postsynaptic active zones of inhibitory as well as excitatory synapses. These filaments intermesh with a lattice of 8-9 nm microfilaments, tentatively identified as f-actin, that is concentrated under the postsynaptic density. It was postulated that the meshwork of 4 nm filaments anchors receptors to the adjacent micro-filament lattice; this extended postsynaptic complex may limit the mobility of receptors and help maintain the curvature of the postsynaptic membrane.

This work has been accepted for publication by the Journal of Cell Biology.

d. Primary auditory terminals in the rostral anteroventral cochlear nucleus form a large calyceal terminal surrounding one pole of the spherical neurons. Synapse formation in this region must be selective (1) to permit the association of only a single terminal with a single neuron (2) to restrict this terminal to a particular region of the cell and (3) to maintain the tonotopic organization of the fibers in the region. This study identified discrete cellular events which may facilitate this developmental specificity. Initial contacts are established randomly with dendritic processes in the neuropil. The formation of these contacts is followed by a proliferation of long appendages from the base of the dendrites which surround a single primary terminal in the neuropil. The terminal then sends out processes which extend along these appendages to contact the dendritic pole of the cell. The appendages retract as axosomatic contacts are formed on this region cell body. A part of this study required the development of computer programs for morphometric analysis of the data. Loss of personnel necessitated abandoning this useful approach to data analysis before all the necessary computer programs were completed.

The intramembrane particle specialization appears in the membrane simultaneously with the arrival of the primary terminal on the cell body. The initial specialization is much larger than that of the mature synapse and the concentration of its intramembrane particles less than that in the mature junction. The reduction in size of the aggregates correlates with a decrease in the size of the postsynaptic density seen in thin sections. The reduction in size of the junction appears to be characterized both by removal of intramembrane particles and by the concentrating of particles in the specialization. This maturation process is complete at the same time that auditory evoked activity is first observed in the cochlear nucleus.

Corresponding to these findings, three manuscripts are in final draft form and will be submitted for publication.

II a. Experiments were conducted in cat anteroventral cochlear nucleus comparing the actions of strychnine and bicuculline on amino acid-induced depression of spontaneous and evoked firing. Strychnine reduced glycine, taurine and  $\beta$ -alanine induced responses more than GABA or muscimol-induced responses. These latter responses were sensitive to bicuculline methiodide and methochloride. Responses to single and paired tone bursts were not sensitive to strychnine or

bicuculline. The results indicate that the depressant amino acid receptors are similar to the receptors found in the cat in other brainstem and spinal cord sites, but differ from those found in cerebellum, thalamus and cerebral cortex. The data also indicate that these amino acids are not involved either in the response to tone bursts at characteristic frequency or in the suppression of this response by a second higher, frequency tone.

These findings have been submitted for publication.

Side band tone bursts inhibited the amino acid-induced firing. Side band tones 1/2 to 3/4 octave above CF and 30 to 45 dB above the threshold at CF usually produced an inhibition in the absence of any excitatory response. Typically, side band frequencies above CF but well within the tuning curve evoked an excitatory response when presented alone or in the presence of DL-homocysteate. The period of decrease in the amino acid-induced firing is most likely due to a postsynaptic inhibition, rather than an after-hyperpolarization or a decrease in excitatory synaptic input, because of the negligible spontaneous activity and the lack of acoustically-evoked excitation at this frequency.

Side band tones which inhibited the amino acid-induced firing also suppressed the activity evoked by a CF tone burst (two-tone suppression). However, not all side band tones that produced two-tone suppression inhibited the amino acid-induced excitation. Side band tones at the border of the tuning curve often produced a near maximum two-tone suppression. Because the two frequencies producing maximum two-tone suppression elicited excitatory or negligible responses, but not inhibitory responses when presented separately, the suppression is most likely due to a decrease in excitatory synaptic input to the NPP units.

These findings have been submitted for publication.

Peak 1 of the BAEP of the cat is shown not be affected by intravenously applied baclofen (2-3 mg/kg). Peaks 2, 4 and 5, however, are strongly suppressed or blocked.

These findings have been submitted for publication.

III. These studies showed a differential solubility of rapidly transported proteins. At pH 8:1, 1% DOC solubilized essentially all labeled protein. However, at pH 7.5, 1% DOC selectively retained proteins with molecular weights of 140,000, 55,000 and 25,000 daltons. Using TX similar results were obtained at both 7.5 and 8.1. Bands that were retained by DOC were enriched by the TX, but several other proteins also were present. Re-extraction had no effect. Control conditions such as buffer alone or with 1.5 M NaCl caused very little solubilization.

Proteins which are insoluble in non-ionic detergents or weak ionic detergents generally are those which are part of a closely linked protein network. An example of this is the postsynaptic density (PSD). In fact, PSD's are purified by solubilizing most other proteins in DOC or triton. The above results on the detergent solubility of rapidly-transported proteins raised the possibility that the insoluble proteins may be associated with the PSD. To explore this possibility, PSD's were isolated from superior colliculi labeled as described above



using the method of Siekewitz which employs TX for solubilization. Under these conditions label was found in the PSD fraction, but showed no distinct pattern. However, a fraction at a higher density specifically showed an enrichment of a 140,000 dalton protein and a protein with a molecular weight greater than 200,000 daltons. Also present were the proteins of molecular weights of 25,000 and 55,000 daltons. Isolating PSD's using the method of Matus which employs DOC gave similar results.

IV. a. The cochlear nucleus in both mice is divisible into three parts: the anteroventral, posteroventral, and dorsal nuclei. Nine cell types can be recognized in the normal mouse. In the anteroventral nucleus spherical cells occupy the rostral pole. Globular cells are located caudally and extend to the interstitial region of the anteroventral nucleus. In the posteroventral nucleus multipolar cells are located rostrally and dark-staining cells occupy the caudal pole. Multipolar cells are also present in the anteroventral nucleus and in the deep region and molecular layer of the dorsal cochlear nucleus. The dorsal and lateral aspects of the ventral nuclei are covered by a granule cell layer. The dorsal nucleus consists of superficial molecular and pyramidal layers and a deep region. The deep region contains small and giant cells as well as multipolar cells. The pyramidal layer is made up of pyramidal cells, horizontal cells and granule cells. Small cells are also present in the molecular layer and throughout the ventral nuclei.

The dorsal cochlear nucleus of the reeler mutant mouse is disorganized and the molecular layer is reduced in thickness. The organization of the pyramidal layer is disrupted with granule cells superficial to pyramidal and horizontal cells. Cells which appear to be homologous to pyramidal cells are also present in the deep region of the dorsal nucleus. The total number of granule cells is reduced by an average of 42% over the whole nucleus and the reduction in granule cells is greatest in the granule cell cap covering the dorsal and lateral surface of the ventral cochlear nuclei. The cytoarchitecture of the ventral cochlear nucleus appears normal.

These findings have been published.

b. In Nissl-stained preparations of the cochlear nucleus there are nine recognizable cell types. These cells are born during three periods of histogenesis prenatally. On gestation days 10.0, 10.5, and 11.0 the pyramidal, giant, and dark-staining cells are born. The spherical, globular, multipolar, and horizontal cells are formed on gestation days 12.0, 12.5, and 13.0 and small cells follow on gestation day 14.5. The onset of granule cell formation is gestation day 14.5 and continues to birth on gestation day 19. At birth, and for at least the first 2 postnatal weeks, glial cells are born. There are no regional gradients in cell birth dates, cells from all birth dates being intermixed. Cell birth proceeds in an orderly sequence that is related only to cell size. Although there were no apparent spatiotemporal patterns, some clustering of labeled cells was evident. These observations do not support the hypothesis that Golgi Type I cells precede Golgi Type II cells in their order of birth since both large and small neurons project beyond the nucleus. There is, nonetheless, a sequential pattern in the onset of cell birth for the auditory system, with cochlear nucleus neurons preceding cochlear neurons.



These findings have been published.

c. The reeler mutant mouse differs from the normal in that there are no AChE-positive fibers in the dorsal nucleus, very few in the granule cell layer covering the lateral aspect of the ventral nucleus, and the number in the posteroventral and caudal anteroventral nuclei are greatly reduced. Perisomatic terminals, small and large terminal boutons, and boutons en passant are present in both normal and reeler mutant mice. AChE-positive cell bodies are found in the deep region of the dorsal nucleus, the rostral part of the posteroventral nucleus, and the anteroventral nucleus of both normal and reeler mutant mice. The observations in the normal mouse are compared to the distribution of AChE-positive fibers and the types of terminals formed by noncochlear afferents in other species. Several possible causes of the lesion of the AChE-positive fibers in the reeler mutant mouse are considered.

These findings have been published.

#### Significance to Biomedical Research and the Program of the Institute:

One of the goals of our work on the auditory nerve has been to fully characterize a possible glutamergic/aspartergic neuron and apply our findings to identify and characterize other neurons using glutamate and aspartate as neurotransmitters. One major drawback in all such studies, not only in ours, has been the lack of a specific method for identification of neurons using these amino acids as neurotransmitters. The findings of our immunocytochemical studies make two major points: They provide a direct visualization of AAT and glutaminase in terminals, fibers and ganglion cells of the auditory nerve. Although the biochemical evidence strongly suggested that these enzymes and glutamate and aspartate are concentrated in terminals and fibers of the auditory nerve, biochemical studies cannot be used to precisely locate these substances. 2. Our studies of the localization of AAT- and glutaminase-like immunoreactivity in auditory nerve terminals in the cochlear nucleus and in neurons of other regions of the CNS in which glutaminase/aspartate may be neurotransmitter indicate that AAT and glutaminase may serve as markers for such neurons.

Our new immunocytochemical findings, our findings on baclofen (under II c.) and our previously reported data from this Laboratory together continue to make the auditory nerve synapse in the cochlear nucleus the best characterized putative glutamate/aspartate synapse in the mammal. This represents a major advance in biomedical research.

We are also the first to introduce an immunocytochemical study of the distribution of AAT- and glutaminase-like immunoreactivity in the CNS, also a major advance in biomedical research.

The Laboratory has adapted a technical innovation used to study the cytoskeleton to identify the components of the cytoskeleton in the postsynaptic complex. The resulting data represent a unique pioneering approach to understanding this structure and its relationship to postsynaptic receptors. The study was correlated with a freeze-fracture study of the development of the postsynaptic membrane specialization. The freeze-fracture study is the first study

of its kind in the central nervous system. The study identified important trophic interactions during the development of the synapse which are amenable to further experimental analysis to determine their basic implications for neuroembryology.

Several recent studies have shown that a cell's cytoskeleton can be isolated by treatment with detergent. The cytoskeleton appears resistant to mild detergent treatment. The resistant proteins are typical cytoskeletal proteins including actin, filaments etc. However, in some cases, integral membrane proteins are also included. This might suggest that they are directly associated with the cytoskeleton. An example is 5'-nucleotidase [Nature 289, 139-144 (1981)]. Most rapidly-transported proteins are believed to be integral membrane proteins. Our axon transport results suggest that the rapidly transported proteins resistant to detergent may be associated with the presynaptic cytoskeleton. A recent paper [Febs. Lett. 124, 289-292 (1981)] describes a 140,000 dalton surface glycoprotein in fibroblasts which is resistant to detergent extraction. It is possible the protein is similar to the 140,000 dalton rapidly transported proteins.

Baclofen, a commonly used antispastic drug, is believed to block the release of excitatory amino acid transmitters. Auditory distortion is one side effect of this drug. Our new results on baclofen not only add to the previous evidence that glutamate/aspartate act as auditory nerve neurotransmitter but also indicate a basis for the clinical auditory side effect of the drug.

The new data provided by the Laboratory on the organization and development of cells and auditory nerve synapses of the cochlear nucleus have to be considered for the understanding of the function of the auditory nerve in health and in disease.

#### Proposed Course:

The study through immunohistochemical methods of the localization at the cellular and subcellular level of the enzymes AAT and glutaminase, both closely associated with the metabolism of glutamate/aspartate, will be continued.

The electrophoretic pharmacological/physiological studies of cells receiving auditory nerve synapses in the cochlear nucleus will be continued. An attempt to further define the possible glutamergic/aspartergic nature of these synapses and of the inhibitory input of such cells will be made.

Experiments on brainstem auditory evoked potentials of the cat will be continued in an attempt to find further new correlates to clinical phenomena.

Loss of six personnel combined with the ongoing hiring freeze precluded further planning.

#### Publications:

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# ANNUAL REPORT

October 1, 1980 through September 30, 1981

## Infectious Diseases Branch

National Institute of Neurological and Communicative Diseases and Stroke

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## ANNUAL REPORT

October 1, 1980 through September 30, 1981

Infectious Diseases Branch, IRP  
National Institute of Neurological and  
Communicative Disorders and Stroke

John Louis Sever, M.D., Ph.D., Chief

### I. RESPONSIBILITY OF THE BRANCH

The responsibility of the Infectious Diseases Branch is to carry out planned, coordinated research programs concerned with infections which damage the human nervous system. The Branch is divided into four sections: 1) Immunochemistry and Clinical Investigations; 2) Experimental Pathology; 3) Neurovirology; and 4) Electron Microscopy. These sections utilize the techniques of immunology, clinical investigations including human volunteers and clinical trials, experimental pathology with nonhuman primates, virology, bacteriology, mycoplasmaology, neurovirology, human tissue culture and electron microscopy.

### II. PROGRAM SEGMENTS

The program segments are: a) perinatal; b) acute; and c) chronic. In each segment we are concerned with: 1) etiology and diagnosis; 2) treatment; and 3) prevention.

The research areas in the program segments include:

#### A. Perinatal

Develop and utilize large scale methods to study the relation between viral, bacterial, mycoplasmal and protozoal infections in the perinatal period and birth defects, related abnormalities and pediatric neurological diseases. Investigate approaches to early diagnosis, treatment and prevention using combined laboratory and clinical studies.

#### B. Acute

Investigate agents which may be responsible for acute neurological diseases such as meningitis, encephalitis, Reye's syndrome, Bell's Palsy, and tic douloureux as well as possible methods for rapid diagnosis, treatment and prevention.

#### C. Chronic

Study chronic neurological diseases such as multiple sclerosis, amyotrophic lateral sclerosis, progressive multifocal leukoencephalopathy, Parkinson's disease, subacute sclerosing panencephalitis, Alzheimer's and Pick's disease and epilepsy using combined tissue culture, immunological, serological, genetic, electron microscopic and clinical approaches for possible infectious etiologies. Whenever possible, explore methods for early diagnosis, treatment and prevention.

### III. SECTION ACTIVITIES

#### A. Section on Immunochemistry and Clinical Investigations (ICI)

##### 1. Perinatal

The Section is responsible for the research and the analysis of Collaborative Perinatal Project sera and data for infection in 60,000 pregnancies. The approaches being used include: 1) clinical infections - correlation with pregnancy outcomes; 2) serological investigation of 8,000 abnormal and 8,000 controls; and 3) high IgM among 30,000 children as a method to identify infected children. Highly sensitive ELISA tests are being applied to these studies.

Additional studies include high risk children and infections in relation to neonatal deaths and herpesvirus infections in pregnancy. A study is being conducted to determine the rate of herpes infections in middle class pregnant women.

##### 2. Acute

Patients with neurological complications following acute Epstein-Barr virus infection are being studied to determine the mechanisms leading to central nervous system involvement. Patients who are critically ill are being treated with Acyclovir.

The ELISA tests are being used in studies of CSF and serum patients with a number of different neurological diseases. Group B streptococcal meningitis infections are being studied in patients at George Washington University Hospital and in experimental monkeys in our laboratories. Herpes simplex encephalitis in the Rhesus monkey model is being studied both for means of rapid viral diagnosis and treatment. Treatment protocols with new antiviral agents are being investigated. Following the demonstration by our laboratory of transmission of rabies infection by corneal transplantation in man, we are investigating the incidence and spectrum of organs infected during rabies encephalitis in mice. Attempts are being made to develop a rapid and technically simple method of detecting rabies antigens in such tissues in the hopes of preventing further tragedies.

##### 3. Chronic

Oligoclonal IgG have been found in the CSF of patients with several different types of neurological diseases including MS, Epstein-Barr virus infection and myasthenia gravis. Specific tests for antibody are in progress using the new micro-oligoclonal method. Special serological investigations of MS and ALS patients are in progress. Patients with panencephalitis following acquired or congenital rubella infection are being studied to determine the pathogenesis of chronic nervous system infection.

Using a new Flow Cytofluorograph technique, studies are underway to define the immune responses in MS and other neurological diseases.

## B. Section on Experimental Pathology (EP)

### 1. Perinatal

This Section is conducting studies using nonhuman primates as models to investigate the effects of in utero infection of several common human pathogens. Current agents include cytomegalovirus (CMV), Venezuelan equine encephalitis (VEE) and western equine encephalitis (WEE) viruses. A model for congenital toxoplasmosis is in the developmental stages. A model for this disease would allow us to investigate new treatments for this pathogen.

### 2. Acute

New methods of treatment and prevention of Group B streptococcal meningitis are being studied using the monkey model developed in this section. Acute encephalitides induced by herpes type I and a varicella like virus, the "Delta Agent", are continuing to be investigated.

### 3. Chronic

Studies of subacute sclerosing panencephalitis in monkeys are in progress. Mechanisms by which the latent viral infection produced by the varicella-like "Delta Agent" can be reactivated and rescued are being studied.

The neuro-oncogenic studies continue with the owl and squirrel monkey models inoculated intracerebrally with JC virus, a human polyomavirus.

## C. Section on Neurovirology (NV)

### 1. Perinatal

This section is studying the immunological response to and control of herpesviruses in non-human primate models. Pathogenesis of primate cytomegalovirus and human Herpes simplex viruses in newborn primates following in utero infection is being investigated with particular emphasis on central nervous system involvement.

### 2. Acute

Immunologic studies are conducted on adult patients with acute herpesvirus infections. These include patients with Herpes simplex virus, cytomegalovirus and Epstein-Barr virus infections with CNS involvement. Cellular and humoral immune responses to these viruses are evaluated to determine their role in control of these agents and their role in preventing disease.

### 3. Chronic

Immunologic studies are conducted to determine the role of immune response to viruses in several chronic neurologic diseases. Patients with multiple sclerosis, systemic sclerosing panencephalitis and other neurologic disturbances are included.

The pathogenesis of JC virus in non-human primates and humans is under study. Molecular "probes" to detect JC viral DNA in tumor tissue and in human brain material have been prepared. The organization and function of the viral DNA in these materials is being investigated. The immune response to JC viral and tumor antigens is under study.



Differences between acute and persistent infections are being sought via use of the patas monkey - simian hemorrhagic fever virus model. Virological and immunological techniques are being used to determine the mechanism of elimination of persistent SHF virus infection by superinfection. Physical-chemical differences between acute and persistent strains of SHF virus are being sought by monoclonal antibody and molecular biology techniques. Cellular immunology techniques are being used to elucidate the cellular interactions involved in restricting the immune response and maintaining tolerance of persistent SHF virus infection.

#### D. Section on Electron Microscopy (EM)

The Section on Electron Microscopy studies in vivo and in vitro the biology and structure of nerve cells and how these are affected by neurotropic viruses. The Section performs basic research related to mechanism of viral and demyelinating diseases of the nervous system, balancing its interest between neurobiology and neurovirology.

The Section is also studying myelin-forming cells and the emergence of myelin proteins during development in the nervous system. Models of myelination and remyelination of central axons are used for these studies. The techniques we use include dissociated nerve cell cultures, aggregated CNS cultures, mixed aggregates of CNS cells and Schwann cells, immunocytochemistry in the light and electron microscope, transmission and scanning electron microscopy, and freeze fracture techniques. In addition, the Section has been recently using videointensification microscopy for the studies of motility and behavior of normal and viral infected cells.

#### IV. FINDINGS

##### A. Perinatal

##### 1. Detection of Low Levels of Rubella Antibody (ICI)

Our studies have shown that current methods for detecting low levels of rubella antibody are often inaccurate. The use of ELISA methods does not always clarify these problems. Several reference standards are needed to help diagnostic laboratories.

##### 2. Commercial Kits for Rubella Antibody Often Vary in Sensitivity (ICI)

A study of 13 new kits marketed for rubella serology showed considerable variation in the ability to detect antibody to rubella. Some may give false negatives, others give false positives. The kits must be monitored carefully by the users. Better techniques for determining the significance of rises in titers need to be developed. Testing laboratories and physicians need to be educated to the value of holding sera for at least 1 year.

##### 3. Toxoplasmosis rarely produces congenital infection (ICI)

Toxoplasmosis antibody was found in 38.6% of the pregnant women tested. Of these, 2.2% had significant rises. Of 22.84% of women, 42 were in the high risk category and 5 had abnormal children that could be attributed to toxoplasmosis infection.

2. Acquired Toxoplasmosis can be Studied in Patas Monkeys (EP-NV)

The patas monkey is readily infected by oral administration of toxoplasmosis cysts. The course of the infection mimics that in humans and makes this an ideal model to study congenital infections.

5. Western Equine Encephalitis (WEE) Virus Causes Hydrocephalus Ex vacuo in Rhesus Monkey Fetuses (EP)

WEE vaccine virus produced severe neurological damage when fetal monkeys were exposed in utero. The virus arrested brain parenchymal development.

B. Acute

1. Group B Streptococcus (GBS) Mortality Reduced by Antepartum Penicillin Therapy (EP)

Antepartum penicillin therapy in pregnant rhesus monkeys infected intraamniotically with GBS significantly lowers neonatal mortality.

2. Cebus apella Monkey Neonates Exposed to Diethylstilbestrol (DES) In utero develop vaginal adenositis (EP)

Vaginal adenositis comparable to that seen in human newborns exposed to DES was observed in cebus neonates that were exposed to DES in utero.

3. Viral Infections of Nerve Cells (EM)

In vivo, the restricted tropism for neurons of a mutant of the wild JHM strain of a mouse coronavirus.

In vitro, a hepatotropic and a neurotropic mutant of JHM strain have restricted tropism for neurons and the mutant shows abnormal assembly in CNS cells.

A wild measles strain can produce a selective infection of mouse neurons in the process of differentiation and the host cell is responsible for the establishment of virus persistence. Infected neurons appear to be unable to redistribute measles antibody complexes in their surface.

C. Chronic

1. New Micromethod for Oligoclonal IgG (ICI)

A new technique which uses only 50 lambda of CSF has been applied to a variety of neurological diseases. About 90% of those active with MS and SSPE develop oligoclonal bands; about 50% of the patients with transverse myelitis, guillain-Barre and myasthenia gravis also have oligoclonal bands.

2. New Method for Study of Cellular Immune Reactions Using Small Numbers Of Cells (ICI)

A new Flow Cytofluorograph to identify immune responses in as little as  $10^3$  lymphocytes is being applied to studies of CSF lymphocytes and lymphocytes from experimental animals. Determination of helper and suppressor cell activities are being made using monoclonal antibody.

3. MS not associated with Canine Distemper (ICI)

MS patients and matched controls were studied for presence of measles and canine distemper antibody. A correlation between measles and canine distemper antibodies was found. We conclude that the canine distemper antibody detected was cross-related to the presence of measles virus antibody and not related to the etiology of MS.

4. MS Patients Have Immune Complexes and Associated Increased Viral Antibodies In The CSF (ICI)

Immune complexes were found in the CSF of MS patients and the levels correlated with the presence of general viral antibodies in the CSF. However, association between antibodies against measles, rubella, coronavirus, and myelin protein can not be identified.

5. Coronavirus antibodies not associated with the etiology of MS. (ICI)

MS patients and matched controls were studied for the presence of 3 strains of coronavirus antibody. The difference in the quantitative and quantitation levels of coronavirus were not significantly different.

6. Neurological Complications of Epstein Barr Virus Infections (ICI)

Patients with EBV related neurological complications have been shown to have viral antibodies and oligoclonal IgG bands in CSF suggesting that viral antigens are expressed within the CNS. Two patients with life threatening neurological complications following EBV infection have been successfully treated with Acycloguanosine Acyclovir.

7. Rabies Virus Transmitted by Corneal Transplantation: Second Report, Pathological Studies. (ICI)

Rabies virus can be transmitted from human to human by corneal transplantation. A second corneal transplant case of rabies encephalitis has occurred in Paris, France, suggesting the problem may be more prevalent than previously suspected.

Results: Neuropathological studies in the first donor recipient cases have been described.

8. Rubella Panencephalitis Following Required Rubella Infection (ICI)

A second case of rubella panencephalitis occurred in a 14 year old male 12 years following acquired rubella infection. The incubation period for both acquired and congenital infections appears to be 12 years. The child has shown steady but slow progression of his disease over the last 4 years.

9. CNS Manufactures Oligoclonal IgG in Acute Herpes Infection. (ICI)

Using the micromethod for oligoclonal IgG, we have demonstrated that patients with biopsy-proven herpes encephalitis produce oligoclonal IgG. We are currently attempting to determine if we can use this technique to replace biopsy in diagnosis of herpes encephalitis.

10. A latent virus infection reactivated by superinfection with another virus (EP)

The latent varicella like "Delta Agent" virus in patas monkeys is reactivated in the latent carrier by superinfection with another virus.

11. Two Species of New World Primates Develop Neurological Tumors Following JC Virus Infection (EP)

After 36 months following inoculation with human JC Virus, 13 owl monkeys and three squirrel monkeys have developed intracerebral neoplasms.

12. Identification of Different Strains of SHF Virus (NV).

Four naturally occurring strains of SHF virus have been isolated and partially characterized. Two of these strains produce acute infections in patas monkeys, while the others produce long-term, probably life-long persistent infections. Infection of adult patas monkeys with persistent virus strains results in immune tolerance of infection.

13. Elimination of Persistent Infection by Superinfection (NV).

Persistent SHF virus infections of patas monkeys can be cleared by superinfection with acute strains of SHF virus. Clearance does not appear to involve interferon or neutralizing antibody. Preliminary data suggest that clearance is by cellular immune mechanisms.

14. Monoclonal Antibodies to SHF Virus (NV).

Eleven clones of hybridoma cells have been isolated which produce monoclonal antibodies to antigenic determinants of the LVR strain of SHF virus. These clones can be divided into two groups. Clones from one group produce antibody to an antigenic determinant found on three different virion polypeptides, while clones from the other group react with a different antigenic determinant found on only one virion polypeptide.

15. Elevated CSF antibodies to viruses in MS (NV)

Antibodies to measles rubella and Epstein-Barr viruses were detected in the CSF in a higher proportion of MS patients than controls. Though not etiologic, these findings suggest altered immunologic activity occurs in response to several infectious agents.



16. Abnormal immunoregulatory responses to viral antigens in MS (NV)

Some active cases of MS showed abnormal T-suppressor cell induction with measles and rubella viruses. These findings suggest that abnormal immunologic regulation in control of these agents occurs during active MS.

17. Normal natural killer cell function in MS (NV)

Lymphocytes from MS patients in all phases of disease were shown to have normal NK cell activity.

18. Lower response to interferon by NK cells in MS patients (NV)

MS patients in active but not stable phase of disease were found to have an abnormally low response to interferon in the induction of NK cell activity. These findings suggest a receptor deficiency in MS during disease activity.

19. Immune complexes in CSF of active MS disease (NV)

Immune complexes were found in elevated levels in a higher frequency in MS cases with acute exacerbation than at other times.

20. JC virus induced tumors contain JCV DNA (NV)

Homology between JC virus DNA sequences found in brain tumor tissue but not adjacent normal tissue of owl monkeys with a recombinant DNA molecular probe was demonstrated by hybridization. The findings provide evidence for an etiologic association between JC virus and the owl monkey gliomas produced following JCV inoculation into owl monkeys.

21. ELISA assay developed to detect antibodies to JCV (NV)

A very sensitive enzyme-linked immunosorbent assay was developed to detect human antibody to JC virion antigen. The assay was more sensitive and specific for JCV than conventional assay.

22. Formation of myelin (EM)

P2, a peripheral nervous system protein, is also localized in rabbit oligodendroglia and CNS myelin.

The major peripheral nerve glycoprotein, Po, is localized on the cytoplasmic side of the Schwann cell plasma membrane, the outer mesaxons and the major dense line of compact myelin. The Golgi system and some cytoplasmic vesicles of Schwann cells also contain Po.

The myelin associated glycoprotein, MAG, is localized in the Schmidt-Lanterman incisure, the paraodal area and the outer mesaxons of actively myelinating Schwann cells.

In vitro, fibronectin promotes rat Schwann cell growth and motility.



## CONTRACT NARRATIVE

Infectious Diseases Branch, IRP, NINCDS

Fiscal year 1980

Bio Tech Research Laboratories Inc. (NO1-NS-8-2388)

TITLE: Provide Special Tissue Culture Cells and Reagents to NINCDS

Contractor's Project Director: Dr. Anton F. Stewen

Current Annual Level: \$47,500.00

Objective: This is a service contract to produce a variety of cells and reagents not available under other mechanisms for use in the research programs of the Branch.

Major Findings: A number of satisfactory lots of special tissue culture cells have been submitted to the branch for use in our studies of the JC virus in owl monkeys and the study of herpes, CMV and rubella virus in neurological disease.

Significance to the NINCDS Program and Biomedical Research: The cells and viruses produced by this Contract have been utilized in the research programs of the Branch. The reagents supplied have helped to identify the role of the "T" and "t" antigens in tumors of owl monkeys.

Proposed Course of the Project: This contract will be continued for another year.

Publications: None

## CONTRACT NARRATIVE

Infectious Diseases Branch, IRP, NINCDS

Fiscal Year 1981

Microbiological Associates (NO1-NS-9-2324)

Title: Development and Delivery of Antigen, Antisera and Viral Diagnostic Reagents.

Contractor's Project Director: Dr. Gabriel A. Castellano.

Current Funding: \$482,500.00

Objectives: This is a service contract to provide reagents for the Collaborative Perinatal Research and JC papovavirus studies.

Major Findings: A large number of high quality viral diagnostic reagents have been provided. These include antigens for Herpes viruses types I and II, Cytomegalovirus, Measles, Rubella, Influenza and Coxsackie A and B. These antigens are used in an attempt to identify the etiology of perinatal infection. Enzyme-linked immunosorbent (ELISA) tests have been developed for herpes, cytomegalovirus and measles. Preliminary studies on the efficiency of these tests in attempting to determine the etiology of perinatal infections will be made. Reagents for ELISA and hemagglutination tests for the JC virus are being developed.

Significance to the NINCDS Program and Biomedical Research: This contract provides to the Collaborative Perinatal Research Projects consistent reagents which are made under similar protocols with the same cells and strains of viruses. This allows us to test these sera for antibodies with viruses that were prevalent in 1964 -1970. Using similar production techniques, data obtained several years ago can be combined with current data. To date, over 80 publications have resulted from analyses of data from these studies. Many of the reports help establish the frequency of disease, the disease syndrome that develops and provides information on which to base rational therapeutic and preventative measures.

Proposed Course: The contract will be continued for the next year.

Publications: Castellano, G.A., Madden, D.L., Hazzard, G.T., Cleghorn, C.S., Vails, D.V., Ley, A.C., Tzan, N.R. and Sever, J.L.: Evaluation of commercial rubella diagnostic test kits. J. Infect. Dis.; Shekarchi, I.C., Sever, J.L., Tzan, N., Ley, A., Ward, L.C. and Madden, D.: Comparison of hemagglutination inhibition test and enzyme-linked immunosorbent assay for determining antibody to rubella virus. J. Clin. Microbiol. 13:850-854, 1981.

## CONTRACT NARRATIVE

Infectious Diseases Branch, IRP, NINCDS

Fiscal Year 1981

Microbiological Associates: (NO1-NS-9-2318)

TITLE: Preparation and Delivery of Special Tissue Culture Cells, Media and Immunological Reagents.

Contractor's Project Director: Norma Parker

Current Level of Funding: \$99,500.00

Objectives: This is a service contract to provide special tissue culture cells, media and immunological reagents for use by the Branch.

Major Findings: A large lot of pretested fetal bovine serum was obtained for use in cellular immunity studies. This lot of sera was non-stimulated to human lymphocytes. Antigens for use in the various types of cell immunity studies was grown in cells produced with this lot of fetal calf serum in order to reduce non-specific cell stimulation. Large lots of pretested microelisa plates have been obtained. Several large lots of high quality alkaline phosphatase labeled anti-human IgG or IgM have been produced which are significant to NINCDS programs and biomedical research.

Production of antigens for cell immunity studies in pretested media and use of that serum in the test itself reduces the nonspecific reactions. This allows us to determine more accurately the specific reaction. Use of specialized equipment and the knowledge of highly qualified individuals on this contract allows us to be far more flexible in purchase of equipment and hiring of personnel. Thus this contract permits us to obtain good reagents at a reasonable price and to maintain a high commitment to research on neurological disease.

Proposed Course of the Project: The contract will be continued for another year.

Publications: None

CONTRACT NARRATIVE  
Infectious Diseases Branch, IRP, NINCDS  
Fiscal Year 1981

Meloy Laboratories, Inc.: (NO1-NS-7-2375)

Title: Isolated Housing and Care of Animals Used in Several Studies of Infectious Diseases.

Contractor's Project Director: Dr. David L. Sly

Current Annual Level: \$258,000.00

Objectives: To provide isolated housing and care of a colony of nonhuman primates consisting of several genera - example: owls Aotus trivirgatus, squirrels Saimiri sciureus, rhesus Macaca mulatta, patas Erythrocebus patas, cynomolgus Macaca fascicularis. To provide housing and care for rodents, rabbits, guinea pigs and mice as required. The animals on experimental studies are monitored daily and biological specimens are collected as directed by written protocols.

Major Findings:

A. Using the human Polyomavirus (JC virus), we have produced glioblastomas in squirrel monkeys (Saimiri sciureus). The route of inoculation was intracerebral. The earliest sign of neurological involvement occurred 15 months following inoculation. To date, three of ten animals have developed tumors. This proves beyond a doubt that the JC virus produces neurological tumors in nonhuman primates since this is the second species that we have been able to induce neurological tumors in, using the human JC virus. The first was in owl monkeys. It should be reemphasized that this is the first human virus found to produce solid tumors in nonhuman primates.

B. We have developed a primate model for Group B streptococcus (GBS) type III in rhesus monkeys. Using this primate model, penicillin therapy for experimentally produced neonatal meningitis due to intracerebral inoculation of GBS was studied. In one group the mothers prophylactically received crystalline penicillin intravenously two hours prior to delivery. The newborns were allowed to stabilize for 30 minutes following delivery and then were inoculated with GBS. All newborns died in this group. A second group was inoculated with GBS 30 minutes after delivery and then 2.5 hours later were treated with penicillin intravenously every four hours for 24 hours. Additional penicillin was given daily intramuscularly for three days. The neonatal mortality was 40% in this group. It was concluded that although penicillin can be used successfully to treat neonates with meningitis after intracerebral inoculation of GBS, penicillin given antepartum as bolus prophylaxis to the mother monkey was ineffective.

Significance to the NINCDS Program and Biomedical Research: The goal of the NINCDS is to carry out planned, directed, research programs concerned with the diseases which damage the human nervous system. This contract provides the backup source in housing and monitoring laboratory animal models to study perinatal and neurological diseases.

Proposed Course of the Project: This contract will be continued for the following year to provide the isolated housing and care of the colony of nonhuman primates.

Publications: None. Listed in each area of study in Experimental Pathology Section.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01-NS-00402-25-ID
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less) Perinatal Infections Causing Damage to the Child - Collaborative Perinatal Project		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	John L. Sever David L. Madden	Chief Veterinary Director
		IDB, IRP, NINCDS IDB, IRP, NINCDS
Other:	Jonas Ellenberg Anita C. Ley Nancy Tzan Dorothy M. Edmonds	Biostatistician Microbiologist Microbiologist Clinical Nurse
		OB & FS, OD, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS
COOPERATING UNITS (if any) Johns Hopkins University    Univ. of CA, Los Angeles and Kaiser Hospital George Washington University Medical School;    OB & FS, OD, NINCDS		
LAB/BRANCH Infectious Diseases Branch		
SECTION Immunochemistry and Clinical Investigations		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:  6.0	PROFESSIONAL:  1.0	OTHER:  5.0
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  The purpose of this study is to determine insofar as possible the role of <u>perinatal infections</u> in the production of fetal damage. To accomplish this, clinical data and a large number of serial serum specimens have been obtained from the 58,000 women and their children in the <u>Collaborative Perinatal Project</u> . Now that the project is <u>complete</u> , it is possible to study perinatal infections with three main approaches: 1) <u>clinical infections</u> ; 2) <u>subclinical infections</u> detected <u>serologically</u> using abnormals and matched controls; and 3) <u>high risk</u> children with <u>elevated IgM</u> levels. Special investigations included the <u>epidemiology</u> of infections and the frequency of congenital <u>toxoplasmosis</u> . <u>Cooperating units</u> work with the Infectious Diseases Branch to study newborns in high risk nurseries. <u>Serum</u> , <u>IgM</u> volumes, plus clinical findings are being used to identify infected infants at risk for <u>perinatal damage</u> . Specific tests are then <u>developed</u> and <u>applied</u> for identification of the infection. Preliminary data indicate that urinary tract infection during pregnancy was found to increase the risk for abortion, stillbirths, neonatal deaths and prematurity and that congenital toxoplasmosis is rare.		
13 - IDB/IRP		



## Project Description:

**Objectives:** The purpose of this study is to determine insofar as possible the role of infections and immunity in the production of abnormal pregnancy outcomes. To accomplish this, 12 collaborating institutions in the Perinatal Research Study plus two special cooperating groups in separate studies obtained specimens of blood and tissue throughout pregnancy, at delivery, post partum, and at set intervals thereafter. These sera are being tested to determine the antibody responses of the patients during pregnancy and post partum and then to relate this serological information to the clinical data for the pregnancy and child. In addition, serum specimens from the children were obtained at one-year-of-age from 10,000 study pregnancies. Sera, throat swabs and urine specimens were also obtained from approximately 5,000 pregnancies. Placental specimens were obtained from 2,500 pregnancies. In special cases when congenital infection is suspected on the basis of clinical or laboratory findings, throat swabs and blood specimens were obtained from the children. Immunoglobulin determinations were performed with the cord blood specimens from the children and specific antibody determinations are also being made with these specimens.

**Methods Employed:** To accomplish this program, blood specimens were obtained from pregnant women at set intervals throughout pregnancy and post partum. Completeness of the sets of sera is determined at the Serum Center of the Infectious Diseases Branch. Data for the 58,000 patients in the Collaborative Perinatal Research Study show that specimens are available from 94.2% of the patients. An average of five blood specimens is available for each patient. Each specimen consists of four vials with 3 ml of serum in each. For this study then, there are approximately 300,000 serum specimens and almost a million and a half vials of sera. There are an additional 5,000 patients studied to date at the Kaiser Hospital in Los Angeles and approximately 3,000 under study at the Johns Hopkins Medical School in Baltimore, Maryland. All specimens are stored at -20°C until tested; and complete filing record concerning basic patient information and the status of the serum available are maintained through a computer system by the Serum Center of the Branch.

In addition to the serum specimens, serial urine and throat specimens were also obtained on a large majority of the patients in the two special studies. These are being studied for direct virus isolation along with swabs obtained from the children at the time of birth.

To date, approximately 80 publications have resulted from the analysis of the data from these studies. The serological method most frequently employed is the complement fixation (CF) test with the use of viral antigens. The test is very versatile and can be performed rapidly and provides broad coverage for a great many of the more than 130 viruses which are known to be of importance to man. Antigens were prepared for most of these viruses and tests of specificity were conducted with animal sera. In addition to the CF method, hemagglutination inhibition (HI) tests are used for many viral serological determinations. When greater specificity is needed, enzyme-linked immunosorbent (ELISA) and neutralization methods are employed. Indirect fluorescence is also used for some of the studies. Virus isolation is conducted with tissue culture or inoculation of experimental animals.

All tests are reproduced completely and a minimum of 95% agreement within 2-fold variation is required. All sera showing significant changes in antibody, together with any sera which did not reproduce, are tested the third time. We are now completing the study of reported viral, bacterial and protozoal infection in pregnant women in the study. Serological tests are used to document these reports. The data is then correlated with the pediatric findings. Approximately 2,500 cases of reported viral infections, 3,000 bacterial infections and several hundred protozoal infections are under investigation. Clinical data is being abstracted, serological tests are being performed in order to document these infections. There are also approximately 1,200 patients identified with a positive serology for syphilis. These are being studied in detail.

A second approach involves a large scale study designed to investigate infection and immunity in relation to 8,000 abnormal children in the study and 8,000 matched controls. The print-out of abnormal patients has been obtained from the Collaborative Perinatal Research Study and this is being reviewed in detail by nurses and physicians from the IDB for more complete information.

From study records, the specific abnormalities under study include abortions, stillbirths, cataracts, congenital heart disease, neonatal deaths, low birthweight (1,000-1,500, 1,500-2,000 grams), IQ below 50-69, enlarged liver, malformations, retarded gross motor development, retarded fine motor development, hearing deficit in both ears, visual impairment, cranial or peripheral nerve damage, cerebral palsy, delayed motor development, hypotonia with poor deep tendon reflexes, nonfebrile seizures, dyskinesia and ataxia, hearing deficit in one ear and elevated bilirubin. The specimens from the mothers of these children and from the children themselves along with carefully matched controls are being studied for the antibody to each of 11 antigens. These antigens include Influenza A, rubeola, rubella, mumps, Coxsackie B<sub>3</sub>, Coxsackie B<sub>4</sub>, Varicella Zoster (VZ, toxoplasmosis, cytomegalovirus (CMV), Herpes Simplex type I and II. All of these agents are known or suspected to be responsible for damage in the perinatal period. All laboratory work is being performed under code. The data is being analyzed by Dr. Ellenberg. A second phase of this study will involve four additional antigens.

The third approach is to identify the children with elevated IgM levels in the newborn period and then correlate these findings with pregnancy outcome, clinical performance of the child and specific serological tests for IgM antibody. Almost 32,000 cord sera have been tested for IgM antibody and approximately 2,000 show elevated levels. These are now being studied in detail.

### Major Findings:

The problem of the reproducibility of serological antibody tests for rubella and other viruses constitutes a major problem in clinical diagnosis of these infections. ELISA tests for virus antibodies provide an alternate method for comparison titrations.

Data for 28 categories of abnormalities have been tabulated from the Collaborative Perinatal Project. The analysis of this information now permits us to determine the risk associated with a variety of infections during pregnancy.

We have studied 22,845 pregnant women and their children for toxoplasmosis. Among the women 38.6% had detectable antibody and 2.2% had significant increases in antibody levels. Based upon this latter group and IgM tests, 42 patients were considered at high risk. Two of the high risk women had children with probable congenital toxoplasmosis and 3 had stillbirths. Three others had children with abnormalities which may or may not be related to toxoplasmosis.

Significance of the Program to the Institute: The use of new serological techniques for a large group of new viruses provides an opportunity to investigate the diseases caused by viruses which are either difficult to isolate or resistant to evaluation because the clinical effects are delayed until a long time after the infection has subsided. In addition, the availability of new immunologic techniques provides the unique opportunity to detect immunologic deficits and to determine the presence of intrauterine infections on the basis of immunologic response. This data can then be correlated and analyzed as in relation to the possible causes of birth defects. The application of this type of analysis has provided valuable information on infections in relation to abnormal pregnancy outcomes and is constantly giving us new insights into the causes of damage to the central nervous system and possible means of prevention of this and other damage to the developing fetus and newborn.

Proposed Course of the Project: The combined immunologic virologic program will continue during the next year. During that time we will complete the tests for the first two phases of the serological studies. Phase three testing will then be initiated using four new antigens.

The three approaches which are being emphasized include:

1. Publication of the correlation of clinically reported infections in pregnancy with serological findings for the pregnancy, immunologic determinations and pregnancy outcome. These studies should be reported for the most part in the next fiscal year.
2. A special commitment to perform serological tests on 8,000 abnormal pregnancies and 8,000 matched controls using 11 antigens. The abnormal children have been identified and the laboratory is now approximately 80% of the way through the testing. Data analysis is being completed for the first 26 abnormal outcome categories.

3. Special test of IgM levels from 32,000 cord sera from children in the Collaborative Perinatal Research Study and in the cooperative studies. This work provides an index for identifying children with possible congenital infections so that more specific testing can then proceed. These investigations are being tested for specific antibody.

Additional studies of high risk groups will be conducted for infections with cytomegaloviruses and herpesviruses.

#### Publications:

Sever, J.L. Sarampo e panencefalite subagude esclerosante. Rev. bras. clin. terap. 9:330-340, 1980.

Sever, J.L. Rubella serology: A need for improvement. Obstetrics & Gynecology 56:127-128, 1980.

Sever, J.L. Infectious Causes of Human Reproductive Loss. Embryonic and Fetal Death. 169-175. Academic Press, New York. 1980.

Poskanzer, D.C., Sever, J.L., Terasaki, P.I., Prenney, L.B., Sheridan, J.L. and Park, M.S. Multiple sclerosis in the Orkney and Shetland Islands, V: The effect of viral titres of histocompatibility determinants. J. of Epidemiology and Community Health. 34:265-270, 1980.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01-NS-01985-10-ID																														
PERIOD COVERED October 1, 1980 through September 30, 1981																																
TITLE OF PROJECT (80 characters or less) Presence of Viral and Nonviral Antigens or Antibodies in Perinatal and Neurological Diseases																																
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI: David L. Madden</td> <td style="width: 33%;">Veterinary Director</td> <td style="width: 33%;">IDB, IRP, NINCDS</td> </tr> <tr> <td>Other: John L. Sever</td> <td>Chief</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>Mary A. Krasny</td> <td>Microbiologist</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>Aurella Krezlewicz</td> <td>Microbiologist</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>Matti Iivanainen</td> <td>Guest Worker</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>William London</td> <td>Veterinary Director</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>Maneth Gravell</td> <td>Research Microbiologist</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>William Wallen</td> <td>Senior Staff Fellow</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>Lilly Jacob</td> <td>IPA Guest Worker</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td>Lata Nerurkar</td> <td>IPA Guest Worker</td> <td>IDB, IRP, NINCDS</td> </tr> </table>			PI: David L. Madden	Veterinary Director	IDB, IRP, NINCDS	Other: John L. Sever	Chief	IDB, IRP, NINCDS	Mary A. Krasny	Microbiologist	IDB, IRP, NINCDS	Aurella Krezlewicz	Microbiologist	IDB, IRP, NINCDS	Matti Iivanainen	Guest Worker	IDB, IRP, NINCDS	William London	Veterinary Director	IDB, IRP, NINCDS	Maneth Gravell	Research Microbiologist	IDB, IRP, NINCDS	William Wallen	Senior Staff Fellow	IDB, IRP, NINCDS	Lilly Jacob	IPA Guest Worker	IDB, IRP, NINCDS	Lata Nerurkar	IPA Guest Worker	IDB, IRP, NINCDS
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COOPERATING UNITS (if any) University of California, Los Angeles Electronucleonics, Inc. Microbiological Associates, Inc.																																
LAB/BRANCH Infectious Diseases Branch																																
SECTION Immunochemistry and Clinical Investigations																																
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205																																
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SUMMARY OF WORK (200 words or less - underline keywords) Continued efforts have been made to determine the etiological agents associated with Multiple Sclerosis. We have continued to use the <u>direct migration inhibition</u> , <u>lymphocyte cytotoxicity</u> and <u>complement mediated cytotoxic</u> tests to determine the cellular immune response of MS patients and carefully matched controls. We have developed a technique associated with <u>flow</u> cytofluorometry to measure responses in a small number of lymphocytes in an effort to determine the cellular immune response in cerebrospinal fluid cells. We have continued to attempt to develop the <u>ELISA</u> technique to measure antibody against a variety of etiological agents which may be associated with multiple sclerosis or other neurological diseases. We have found that the ELISA technique is as adequate as existing serological tests to determine antibody titers but in most cases although the antibody titers are higher, the specificity of the tests are not much greater. Routine monitoring of cultures from experimental viral studies for <u>mycoplasma</u> contamination and efforts to develop new techniques to monitor tissue cultures for contamination have been continued.																																



Project Description:

Objectives: To isolate and identify viral and non-viral antigens and/or antibodies. To utilize these antigens and/or antibodies for more specific, rapid, sensitive identification of antigens and antibodies and/or a more accurate identification of infectious agents in diseases. To define the humoral and cellular immune response of patients with neurological disease to these antigens. To determine the relationship between specific infectious agents in pregnant women and mental retardation, congenital jaundice and postnatal jaundice.

Methods Employed: Human culture lines chronically infected with subacute sclerosing panencephalitis (SSPE) measles virus, herpes simplex virus types I and II (HSV-I and II), and cytomegalovirus (CMV) have been used to determine the immunological response of SSPE, and multiple sclerosis (MS) patients and matched pal controls to these antigens. The infected cells have been labeled with  $^{51}$  Chromium. Target cells and various concentrations of lymphocytes or serum are reacted for 18 and 24 hours and the amount of  $^{51}$  Chromium specifically released is determined.

We have adapted flow cytofluorometric techniques to measure the cellular immune response of patients with multiple sclerosis. The amount of DNA and RNA in cells using the cytofluorograph has been determined by vital staining of cells in a one step method using acridine orange. The cells are stained for 8 minutes and then passed through the cytofluorograph. Analysis of the ratio of DNA to RNA present in the cell is displayed using a single phase pulse height analysis. The amount of change in DNA in specific channels is compared with the control and a stimulation index determined. The analysis takes about 2 minutes for each sample. Efforts are being made to determine the immune response capabilities of individuals using lymphocyte cytoplasmic antigen markers. Cells are treated with flourine labeled antibody prepared in hybridoma septers. The frequency of cells with antigen markers in patients with neurological diseases is compared with that in normal conditions.

Adaptation of the enzyme-linked immunosorbent assay (ELISA) to detect antibody against several viruses associated with neurological diseases has been accomplished. The viral antigens are absorbed onto disposable plastic plates, treated with serum and the unbound antibody is then washed off. Anti-human IgG conjugated to phosphatase is reacted to the serum remaining in the plate, excess washed off, substrate added and the color which develops is proportional to the amount of antibody in the unknown sera. Correlation between existing assays are very close. Efforts are being initiated to identify viral antigens using plates coated with specific antiviral antibodies.

Virus preparations and antigens used in cellular immune studies have been examined for presence of mycoplasma. Comparison of several methods for identification of mycoplasma and tissue culture are underway. Techniques to detect nonculturable strains have been adapted. The emergence of additional strains that do not grow in liquid media makes these studies extremely important.

Major Findings: Further studies on the roles of viruses in the etiology of Multiple Sclerosis have been completed. Our investigation of patients and controls from the north central United States has been extended to populations in California and Faeroes Islands. In all populations there was a significant increase in the mean titer of measles antibody in the serum of MS patient population as compared to the control population. There was no increase in the serum titer of other viruses tested such as cytomegalovirus, herpesvirus types I and II or vaccine virus in the MS population as compared to the control population. In the populations studied when carefully matched patients as to age, sex, ethnic background and place of residence were studied, there were no differences in frequency of the HLA genotype between the MS and control patients. No differences in the cellular immune responses of MS patients or matched controls were detected. In the California study, the MS patients with DW2 antigens had a different humoral and cellular immune response to measles virus than those without. In the north central population, this difference was not evident. Studies have been completed on the frequency of coronavirus and canine distemper antibodies in MS patients and matched controls. From our studies, coronavirus antibody is no more frequent in MS patients than controls and it is felt that coronavirus is not related to MS. Canine distemper antibody is more frequent in MS patients than controls, however, as the distribution of canine distemper antibody and measles antibody is similar and the two viruses are serologically related, we consider that canine distemper virus is not associated with MS.

Utilizing the antibody survey techniques, efforts have been made to determine the etiology of Parkinson's, amyotrophic lateral sclerosis and late onset post-poliomyelitis progressive muscular atrophy. There was no significant serological evidence to suggest a significant association with a persistent or post virus infection.

Studies completed indicate that the cellular immune responses  $2 \times 10^3$  cells culture can be readily detected with the cytofluorograph. The response of cells at 3 days is similar to that observed using conventional techniques which utilize  $5 \times 10^5$  cells/cultures. The amount of phytohemagglutination (PHA) time and volume being similar eliminates the effect of secondary stimulation. Observations indicate that the cytofluorograph can measure the cellular immune response as early as 24 hours. Using the PHA technique, parameters for developing the measurement of non-specific cellular immune responses in non-human primates have been developed. Efforts are underway to correlate immunosuppression with quantity and quality of *in vitro* lymphocyte response. Studies to determine the immune surface antigens have just been initiated. Currently, information seems to indicate that variables in the frequency of cellular make-up of cells is great. How this affects the results remains to be seen.

Measurement of antibody against several common viruses in the serum and cerebral spinal fluid is possible utilizing the ELISA technique. A comparison of the serum/cerebral spinal fluid ratio indicates that log ratios under 2 were consistent with CNS in suite product of antibody. Presence of in suite antibody production was also related to oligoclonal antibody production.

A micromethod to detect oligoclonal IgG from 50  $\mu$ l of unconcentrated cerebral spinal fluid was developed using polyacrylamide gel electrophoresis in sodium dodecylsulphate (SDS-PAGE). This technique is as sensitive as the agarose electrophoresis technique which requires up to 5 ml of cerebral fluid which must be concentrated 50-100 times. The small volume of unconcentrated CSF required enhances the usefulness of the test. The addition of enzyme-labeled antibody to the developed PAGE strip has demonstrated that the band is indeed gamma globulin. Studies utilizing these strips are being undertaken in an effort to determine the specificity of the antibody.

Cerebral spinal fluid from several types of diseases have been studied for the presence of oligoclonal IgG. The largest number of bands appear to be in those chronic diseases which are thought to be associated with infectious agents such as SSPE and MS. A large number of patients with acute viral encephalitis have oligoclonal bands during acute phase (herpes, measles, EBV) but after the disease has cleared these bands disappear. The chronic, non-classical viral diseases - Kuru -Scrapie, do not have multiple bands.

Studies of tissue culture levels, seed viruses and media for mycoplasma contamination continues. Continued monitoring of common reagents used by the Infectious Diseases Branch is necessary to prevent these contaminating agents from producing artifacts in the data. Efforts to develop new and more rapid techniques for identifying these agents continue.

Significance of the Program to the Institute: The development of more specific antigens or antibodies which measure more accurately the immunological status of an individual is needed. Highly specific antigens or antibodies may help identify the biological differences between pathogenic and nonpathogenic strains of these organisms and identify the etiology of obscure diseases.

Proposed Course of the Project: Further studies will be done to identify the antigens associated with the measles and rubella, HSV-I and II and CMV. Cellular and humoral immune studies are being expanded in an effort to detect small amounts of antigen on intact cells and immunological response differences which may account for neurological diseases.

Kumar, A., Selim, M.S., Madden, D.L., Wallen, W.C., Vasquez, H.H. and Nankervis, G. Humoral and cell-mediated immune responses to herpesvirus antigens in patients with cervical carcinoma. Gynecol. Oncol. 10: 18-25, 1980.

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Madden, D.L., Wallen, W.C., Houff, S.A., Shekarchi, I.C., Leinikki, P.O., Castellano, G.A. and Sever, J.L. Measles and canine distemper antibody. Presence in sera from patients with multiple sclerosis and matched control subjects. Arch. Neurol. 38, 13-15, 1981.

Marttila, R.J., Rinne, U.K., Halonen, P., Madden, D.L. and Sever, J.L. Herpesviruses and Parkinsonism. Herpes simplex virus types 1 and 2, and cytomegalovirus antibodies in serum and CSF. Arch. Neurol. 38, 19-21, 1981.

Iivanainen, M., Leinikki, P., Taskinen, E., Shekarchi, I.C., Madden, D. and Sever, J.L. CSF oligoclonal bands, immunoglobulins and viral antibodies in progressive myoclonus epilepsy. Arch. Neurol. 38, 206-208, 1981.

Madden, D.L., Wallen, W.C., Houff, S.A., Leinikki, P.A., Sever, J.L., Holmes, K.A., Castellano, G.A. and Shekarchi, I.C. Coronavirus antibodies in sera from patients with multiple sclerosis and matched controls. Arch. Neurol. 38, 209-210, 1981.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  <div style="text-align: right;">Z01-NS-02038-09-ID</div>
PERIOD COVERED <b>October 1, 1980 to September 30, 1981</b>		
TITLE OF PROJECT (80 characters or less)  <b>Combined Clinical, Viral and Immunological Investigations of Acute and Chronic Diseases of the Central Nervous System</b>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
<b>PI:</b>   <b>Other:</b>	<b>John L. Sever</b> <b>Sidney A. Houff</b>  <b>David L. Madden</b> <b>Maneth Gravell</b> <b>Monique Dubois-Dalcq</b> <b>Anita C. Ley</b>	<b>Chief</b> <b>Clinical Associate</b>  <b>Veterinary Director</b> <b>Research Microbiologist</b> <b>Research Microbiologist</b> <b>Microbiologist</b>
		<b>IDB, IRP, NINCDS</b> <b>IDB, IRP, NINCDS</b>  <b>IDB, IRP, NINCDS</b> <b>IDB, IRP, NINCDS</b> <b>IDB, IRP, NINCDS</b> <b>IDB, IRP, NINCDS</b>
COOPERATING UNITS (if any) <b>University of Vermont</b> <b>VA Hospital, Washington, D.C.</b> <b>Georgetown University Medical School, Washington, D.C.</b> <b>Children's Hospital, Washington, D.C.</b>		
LAB/BRANCH <b>Infectious Diseases Branch</b>		
SECTION <b>Immunochemistry and Clinical Investigations</b>		
INSTITUTE AND LOCATION <b>NINCDS, NIH, Bethesda, Maryland 20205</b>		
TOTAL MANYEARS: <div style="text-align: center;"><b>4.5</b></div>	PROFESSIONAL: <div style="text-align: center;"><b>1.5</b></div>	OTHER: <div style="text-align: center;"><b>3.0</b></div>
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  <b>Clinical and laboratory studies are conducted to determine etiology (<u>infection, immunity and/or genetics</u>) for chronic diseases of the central nervous system. Current studies include <u>Multiple Sclerosis, Progressive Multifocal Leukoencephalopathy, Subacute Sclerosing Panencephalitis, Myasthenia Gravis, Amyotrophic Lateral Sclerosis and Parkinson's Disease.</u> Combined clinical data, genetic information, HLA and MLC typing, virus serology and <u>virus isolation studies</u> are obtained for these studies.</b>  <b>Oligoclonal IgG was found in the CSF of 90% of MS patients, 90% of SSPE, 100% of patients with biopsy proven herpes simplex encephalitis and 50% of the patients with Myasthenia Gravis. No unusual antibody levels or oligoclonal IgG patterns were found in the serum or cerebrospinal fluid of patients with Parkinson's disease, Huntington's disease or Creutzfeldt-Jakob disease. Previously unrecognized as a possibility the human-to-human transmission of rabies by corneal transplant was demonstrated. Donors with neurological diseases must be carefully reviewed before their tissue is used in transplantations.</b>		
23 - IDB/IRP		



### Project Description:

Objectives: Clinical and laboratory studies are being conducted on chronic infections of the central nervous system (CNS). During this year, the investigations have centered primarily on multiple sclerosis (MS), progressive multifocal leukoencephalopathy (PML), subacute sclerosing panencephalitis (SSPE), myasthenia gravis (MG), amyotrophic lateral sclerosis (ALS) and Parkinson's disease. These studies have epidemiological, serological, cellular immune, viral and therapeutic components. They involve collaboration of a number of groups throughout the United States.

Methods Employed: MS patients and tissues are obtained from a number of collaborators throughout the world. New tissue culture methods and electron microscopic techniques are used in these studies as well as genetic and cellular immune tests.

Specimens from patients with PML, SSPE, ALS, MG, Parkinson's disease, and neurological complications of Epstein-Barr virus infections as well as several types of brain tumors are being studied virologically and immunologically. Individuals immunized with measles vaccine are being tested for antibody levels and persistence of antibody.

Major Findings: The CSF of 90% of the MS patients, 90% of SSPE, 100% of biopsy proven herpes simplex encephalitis, 43% of encephalitis of unknown etiology, 40% of Guillain-Barre' and 10% of patient with Alzheimer's and other neurological diseases have oligoclonal IgG. Monoclonal or oligoclonal bands appear in 50% of the myasthenia gravis patients. The occurrence of the abnormal IgG in the myasthenia gravis patients suggests that CNS involvement is more complex and more extensive than has been previously recognized. Serum and cerebrospinal fluid from patients with Parkinson, Huntington's, Creutzfeldt-Jakob disease, ALS and progressive muscle atrophy have been tested and no significant increase in the antibodies against a variety of viruses or oligoclonal IgG have been recognized.

Patients with neurological complications of EBV infections have been shown to have antibodies to early antigens and viral capsid antigens in the CSF. Oligoclonal bands have been shown to occur during the active stages of disease and disappear as the disease process resolves. One patient has experienced exacerbations of her illness with the reappearance of antibodies in CSF. These findings suggest the expression of EBV antigens within the central nervous system and may serve as sensitive diagnostic indicators for neurological illnesses associated with EBV infections.

The human to human transmission of human rabies by corneal transplant was demonstrated. The initiating source of infection was not identified although the occupational history of the original case suggests that wildlife exposure was possible. This study points out that donors with neurological disease must be carefully evaluated if their tissues are to be used for transplantation.

Patients with neurological complications or Epstein-Barr Virus Infections have been shown to manifest antibodies to various viral antigens in CSF as well as oligoclonal bands. One patient with encephalitis following infectious mononucleosis has been treated with Acyclovir, a new antiviral agent. The patient recovered.

A clinical protocol for herpes and progressive multifocal leukoencephalopathy has been developed. We will attempt to identify the cellular and humoral parameters which are altered prior to infection and those which might be stimulated to increase recovery without severe sequelae. Cooperative studies with National Institute of Allergy and Infectious Disease will be initiated to test the effectiveness of acyclovir and other drugs for viral therapy.

A second case of acquired rubella panencephalitis occurred in a 14 year old male who contracted a rubella virus infection during the pandemic of 1965. Rubella antibody and oligoclonal bands were present in CSF. Cerebellar biopsy revealed loss of molecular layers, cells and vacuolization as well as loss of Purkinje's cells. Attempts to rescue rubella virus or identify virus by either electron microscopy or fluorescent antibody have been unsuccessful. The physical and neurological development of this child continues to be monitored. These evaluations indicate that the child continues to degenerate.

Significance of the Program to the Institute: Clinical and laboratory studies of MS, PML, SSPE, MG, postinfectious polyneuritis, ALS, neurological complications of infectious mononucleosis, and Parkinson's disease permit direct investigation of the possible causes of these diseases and provide us with an opportunity to study unique "experiments" of nature which often provide very valuable insight into disease process. These studies are designed to take advantage of both the epidemiology as well as the direct laboratory approaches to the problems of acute and chronic infections of the CNS.

Proposed Course of the Project: Additional studies in attempts to identify the etiology of Multiple Sclerosis, Myasthenia Gravis, ALS and Parkinson's Disease will be continued. Emphasis will be placed upon identifying the role of the Epstein-Barr virus as a cause of neurological disease. A treatment protocol using Acyclovir for patients with complications of EBV infections has been submitted. Continued effort to identify the relationship of PML and brain tumors are being undertaken. Attempts will be made to develop methods of rapid diagnosis for viral encephalitis using a multicenter cooperative study, which has been approved. Research to identify the cause of oligoclonal IgG bands in CSF and the cellular immune response of CNS cells is continuing.

Since the first report of human to human transmission of rabies, a second case has occurred in Paris, France. The frequency of corneal involvement in natural rabies is being investigated in mice. Methods of rapid diagnosis using fluorescent antibody techniques are being developed to aid in determining suitability of individual cases for corneal transplantation. Studies of the pathogenesis of encephalitis and atypical Guillian-Barre' Syndrome due to rabies are being pursued to determine what factor(s) favor the development of one syndrome as compared to the other.

Publications:

Dalakas, M.C., Houff, S.A., Engel, W.K., Madden, D.L. and Sever, J.L. CSF "monoclonal" bands in chronic relapsing polyneuropathy. Neurology. 30: 864-867, 1980.

Taylor, J.R., Selhorst, J.B., Houff, S.A. and Martinez, A.J. Chlordecone intoxication in man I.Clinical observation. Neurology. 28: 626-630, 1978.

Martinez, A.J., Taylor, J.R., Dyck, P.J., Houff, S.A. and Isaacs, E. Chlordecone intoxication in man II.Ultrastructure of peripheral nerves and skeletal muscle. Neurology. 28: 631-635, 1978.

Britton, D.E., Houff, S.A. and Eiben, R.M. Possible interactions between rabies vaccination and a progressive degenerative CNS disease. Arch. Neurol. Letters to the Editor. 35: 693, 1978.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  <b>Z01-NS-01731-13-ID</b>
PERIOD COVERED <b>October 1, 1980 to September 30, 1981</b>		
TITLE OF PROJECT (80 characters or less) <b>Isolation, Characterization and Diagnosis of Infectious Agents from Chronic Diseases</b>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	Maneth Gravell	Research Microbiologist      IDB, IRP, NINCDS
Other:	William T. London	Veterinary Director      IDB, IRP, NINCDS
	Marta Monzon	Visiting Fellow      IDB, IRP, NINCDS
	Olajide Agbede	Visiting Fellow      IDB, IRP, NINCDS
	Jose Luis Sagripanti	Visiting Fellow      IDB, IRP, NINCDS
	Rebecca S. Hamilton	Biologist      IDB, IRP, NINCDS
	Otto Gutenson	Biologist      IDB, IRP, NINCDS
	Blanche Curfman	Biologist      IDB, IRP, NINCDS
	Robert Brown	Biological Lab Technician      IDB, IRP, NINCDS
COOPERATING UNITS (if any)		
LAB/BRANCH <b>Infectious Diseases Branch</b>		
SECTION <b>Neurovirology</b>		
INSTITUTE AND LOCATION <b>NINCDS, NIH, Bethesda, Maryland 20205</b>		
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.0	1.0	2.0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) <p> <u>Superinfection</u> of <u>persistently infected patas monkeys</u> with <u>acute strains of simian hemorrhagic fever (SHF) virus</u> has resulted in elimination of both the persistent and acute viruses, thus clearing the <u>persistent infection</u>. To date, confirmation of this result has been obtained in all 10 persistently infected animals, superinfected by this procedure. Our results suggest that <u>immunoregulatory controls</u> are involved in maintaining persistent SHF virus infections in the patas monkey and that these controls can be over-ridden by superinfection with acute strains of virus.         </p> <p> <u>Neutralizing antibody</u> to persistent strains of SHF virus was not detected in sera of patas monkeys cleared of infection by superinfection. However, a persistent infection could not be re-established in the animals cleared of persistent infection. The mechanism of clearance of persistent infection did not appear to involve <u>interferon</u>. We are currently evaluating whether <u>cellular immune mechanisms</u> are involved in this clearance.         </p>		



### Project Description:

Objectives: To use virological, biochemical and immunological techniques to study persistent viral infections and their role in chronic neurological diseases.

Methods Employed: Clones of mouse hybridomas producing monoclonal antibodies to antigens of the LVR strain of SHF virus were isolated by the method of Kohler and Milstein. The antigens to which these monoclonal antibodies were directed were determined by enzyme-linked immunosorbent assay, immunodiffusion, immunoprecipitation, polyacrylamide gel electrophoresis and fluorography. Rhesus or patas monkey macrophages used for growth or infectivity assay of SHF virus were obtained by peritoneal lavage and purified by centrifugation on Ficoll-Hypaque. Peritoneal macrophages were maintained in vitro by tissue culture techniques. SHF virus was purified by differential and density gradient centrifugation techniques.

Major Findings: Simian hemorrhagic fever (SHF) virus is best known for its capacity to produce an acute hemorrhagic disease which is essentially 100 percent fatal for monkeys of the genus Macaca. It is less well known that strains of this virus can produce an asymptomatic, probably life-long, persistent infection in the patas monkey (Erythrocebus patas).

We have identified and partially characterized four naturally occurring strains of simian hemorrhagic fever virus. All four of these strains produce a fatal hemorrhagic disease in macaque monkeys. In contrast, two of these strains produce an acute, usually non-fatal infection in the patas monkey, while the other two strains produce an asymptomatic persistent infection. Evidence indicates that the target cell for SHF virus infection is of monocyte-macrophage lineage. Our results suggest that the extreme lethality of the hemorrhagic disease in macaque monkeys probably results from the heightened sensitivity of their macrophage population to infection and lysis.

We have focused our efforts on SHF virus infection of patas monkeys because of the natural persistent infection which occurs in this species. Patas monkeys infected with acute strains of SHF virus develop immunity and are free of virus 30 to 60 days after infection. These acute infections induce high titers of viral antibody. Conversely, viral antibody is barely detectable in the serum of patas monkeys in which persistent infections have been established by infection with persistent viral strains. The persistent and acute virus strains have been shown to be antigenically related, but results of enzyme immunoassays indicate that they are not identical. Furthermore, specific antisera to acute strains do not neutralize persistent strains. In fact, neutralizing antibody to persistent strains has not been demonstrated and persistent infections can be established in virus-free animals previously infected with acute strains of virus and possessing high titers of specific antibody. Surprisingly, however, superinfection of persistently infected patas monkeys with acute strains of SHF virus has resulted in elimination of both the persistent and acute viruses, thus clearing the persistent infection. To date, confirmation of this result has been obtained in all 10 persistently infected animals, superinfected by this procedure. These results imply that



immunoregulatory controls are involved in maintaining persistent SHF virus infections in the patas monkey and that these controls can be over-ridden by superinfection with acute strains of virus.

Patas monkeys cured of persistent infection by superinfection were reinfected with the original strain of persistent virus (P-248 strain) about 4 months and 12 months after being cleared of infection. Although virus replication was demonstrated in the reinfected animals, infectious virus was detectable for only about a week. Neutralizing antibody to the P-248 strain of virus was not detected in sera from the reinfected animals. However, memory cells must have been activated in the clearing process. We are currently evaluating whether cellular immune mechanisms were involved in clearing the persistent infection.

The mechanism of clearance of the persistent infection does not appear to involve interferon. Interferon has not been detected in sera from infected animals, nor has twice daily intravenous administration of large doses of human interferon (500,000 units daily/animal) over a two-week period had any measurable effect in clearing persistent SHF virus infection of patas monkeys.

Polypeptides of acute and persistent strains of SHF virus are also being compared by polyacrylamide gel electrophoresis. Five virion polypeptides have been detected in acute and persistent strains of virus. One of these polypeptides is a glycoprotein. Molecular weights of polypeptides from both the acute and persistent strains were similar and range from about 50,000 to 10,000 daltons.

Differences in acute and persistent strains of SHF virus are also being sought by use of monoclonal antibody technology. Eleven clones have been isolated which produce monospecific antibody to antigens of the prototype strain of SHF virus (LVR strain). Immunoprecipitation studies show that several of the clones probably produce antibody to the same antigenic determinant. However, clones which produce antibody to specific antigenic determinants located on different polypeptides have been isolated. These clones are being characterized by immunoprecipitation, enzyme immunoassay, indirect immunofluorescence and neutralization tests.

A persistent SHF virus infection (P-248 strain) has been established in vitro in USU-104 cells. This infection occurs without causing noticeable cytopathology in these cells. The capacity of the P-248 strain to produce a persistent non-lytic infection of USU-104 cells is a very stable trait of this virus. Its extensive serial passage through USU-104 cells (over 50 passages) and rhesus monkeys (six passages) failed to unmask virus with lytic properties for USU-104 cells. Culture medium from persistently infected cultures assayed in rhesus monkey peritoneal macrophage cultures, where measurable cytopathology occurs, was found to contain about  $10^5$  to  $10^6$  TCID<sub>50</sub>/ml of cell-free P-248 virus. Immunolabeling techniques showed only a low percentage of infected cells in persistently infected cultures. Preliminary cloning experiments support this conclusion. The mechanism of persistence of the P-248 strain in USU-104 cells has not been determined, but evidence suggests it does not involve interferon or defective interfering particles.

Significance of the Program to the Institute: A number of fatal neurological diseases are caused by persistent viral infections, including subacute sclerosing panencephalitis, progressive multifocal leukoencephalopathy, cytomegalovirus inclusion disease, rubella panencephalitis, etc. Usually, irreversible damage has occurred in patients with these diseases before their cause is determined and little hope remains to arrest fatal progression of the disease. Thus, emphasis must be placed on learning how persistent infections become established, evade elimination by host immunological defenses and cause pathological damage to the host. This is the long term goal of this project.

Proposed Course of the Project: We will continue to isolate clones of hybrid cells producing monoclonal antibodies to antigens of acute and persistent strains of SHF virus. These monoclonal antibodies will be used to characterize antigenic differences between the various virus strains, to determine whether the target cells differ in acute and persistent infections, and to clarify the virus-cell interactions, receptors and signals involved in host tolerance of infection. In addition, studies will be initiated using recombinant DNA technology to determine genomic differences between acute and persistent strains of virus.

Publications:

Gravell, M., Palmer, A.E., Rodriguez, M., London, W.T. and Hamilton, R.S.: Method to detect asymptomatic carriers of simian hemorrhagic fever virus. Laboratory Animal Science 30:988-991, 1980.

Gravell, M., London, W.T., Rodriguez, M., Palmer, A.E. and Hamilton, R.S.: Simian haemorrhagic fever (SHF): New virus isolate from a chronically infected patas monkey. J. General Virology 51:99-106, 1980.

Gravell, M., London, W.T., Rodriguez, M., Palmer, Amos E., Hamilton, Rebecca S. and Curfman, Blanche L.: Studies on simian hemorrhagic fever virus infection of patas monkeys: I. Serology. In: Proceedings of the Symposium on the Comparative Pathology of Zoo Animals. Smithsonian Institution Press, Washington, DC, 1980, p. 167-170.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  <div style="font-size: 1.2em; font-weight: bold;">Z01-NS-01983-10-ID</div>
PERIOD COVERED <div style="font-weight: bold;">October 1, 1980 to September 30, 1981</div>		
TITLE OF PROJECT (80 characters or less) <div style="font-weight: bold;">Chronic Viral Infections</div>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	William C. Wallen	Senior Staff Fellow IDB, IRP, NINCDS
Other:	David L. Madden	Veterinary Director IDB, IRP, NINCDS
	John L. Sever	Chief IDB, IRP, NINCDS
	William T. London	Veterinary Director IDB, IRP, NINCDS
	Sidney A. Houff	Clinical Associate IDB, IRP, NINCDS
	Renee G. Traub	Microbiologist IDB, IRP, NINCDS
	Nancy Miller	Expert Consultant IDB, IRP, NINCDS
	Eugene Major	IPA IDB, IRP, NINCDS
COOPERATING UNITS (if any) Microbiological Associates, Bethesda, MD    Loyola University, Maywood, IL George Washington University Medical School, Washington, DC Veterans Administration Hospital, Washington, DC Georgetown University Medical Center, Washington, DC		
LABORATORY <div style="font-weight: bold;">Infectious Diseases Branch</div>		
SECTION <div style="font-weight: bold;">Neurovirology</div>		
INSTITUTE AND LOCATION <div style="font-weight: bold;">NINCDS, NIH, Bethesda, Maryland 20205</div>		
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
1.8	0.8	1.0
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) <p>           Studies with multiple sclerosis (MS) have shown <u>elevated antibody levels</u> in CSF to measles virus, rubella virus and Epstein-Barr virus related antigens but not to Herpes simplex virus, cytomegalovirus or influenza type A virus. <u>Abnormal induction</u> of T-suppressor cells was found in response to measles virus in some MS patients during active disease. Normal activity of the <u>natural killer</u> (NK) cells was found but a lowered ability to respond to interferon was shown to occur in the NK cell cytotoxicity assay by lymphocytes from MS patients with active disease. <u>Immune complexes</u> were detected in CSF from active MS cases and highest levels were found in patients with acute exacerbation of disease. Abnormal <u>immunoregulatory functions</u> were demonstrated in MS cases and found to be temporarily associated with exacerbation of disease.         </p> <p>           JC virus (JCV) DNA has been detected in <u>brain tumors</u> produced in owl monkeys following inoculation with JCV. An enzyme linked immunosorbent assay has recently been developed to detect human antibody to JCV.         </p>		

### Project Description:

**Objectives:** This project investigates clinical and biological significance of viral infections in the causation of chronic neurological diseases. The varied immunologic responses to viral infections are studied to determine which parameters control an infection and which contribute to persistence of the virus and result in disease. Particular emphasis is placed on infections by herpesviruses, types I and II (HSV-I, HSV-II), cytomegalovirus (CMV) and Epstein-Barr virus (EBV) and polyomaviruses (JCV and BKV). Cell-mediated immunity appears to play a critical role in controlling these viruses. However, the relationship of immunity and its regulation to chronic, persistent infections is not understood. Therefore, we plan to study the immune response and its regulation to these viruses during chronic and acute neurologic diseases.

**Methods Employed:** The principal methods employed in the study of chronic viral infections of humans include: 1) virus isolation, latent genome recovery by cocultivation, chemical or mitogenic activation; 2) detection of viral genome using cloned, recombinant DNA "probes" for detection of viral DNA sequences in tissues by molecular hybridization; 3) virus quantitation and identification by serology, host cell cytopathogenic sensitivity spectrum and fluorescent antibody techniques; 4) large scale serological surveys of materials from selected patients with specific disease entities using fluorescent antibody assays, enzyme-linked immunosorbent assays (ELISA) and antibody dependent lymphocytotoxicity assays; 5) cell-mediated immunity (CMI) is detected employing the lymphocyte stimulation assay for measuring effector cell functions or antigen recognition and the direct cytotoxicity or immune interferon assays for measuring effector immune mechanisms; 6) immune regulation studies will be performed using a suppressor T-cell assay recently developed to evaluate the regulatory function during viral infections which result in neurologic disease; and 7) the role of natural killer cells and its amplification by immune interferon and the effect of immune complexes will be studied to determine their contribution to pathogenesis or disease control.

### Major Findings:

a. **Herpesviruses** - Occasional cases of multiple sclerosis (MS) were found with CSF antibody to Epstein-Barr virus (4/52) but none to Herpes simplex or cytomegalovirus during active phase of disease. Abnormal cellular immunity and immunoregulatory functions directed against EBV antigens were not demonstrated nor were they found to occur with antigens of the other herpesviruses (HSV or CMV).

b. **Papovaviruses** - In a collaborative study with Walker, Padgett and Zu Rhein (University of Wisconsin), we have successfully produced glioma brain tumor in adult owl monkeys inoculated intracerebrally with JC virus. We have established a brain tumor production rate of approximately 30% after 30 months in these animals. Mean tumor induction time was about 22 months. The tumors are predominantly glial (glioma/astrocytoma) in nature and tend to grow as localized, large encapsulated tumor. The tumors have been detected by computerized axial tomography (CAT) scan at approximately 1 cm in size (Di Chiro et al.).



Replication of JC Virus Expression in vitro - Fresh brain tumors from JC inoculated monkeys readily established cell lines in vitro. Cultured tumor cells initially contain JC related T antigen as detected by immunofluorescence. However, upon passage, the incidence of T antigen positive cells decreases from 1-5% at initiation to an undetectable level. More recent work in collaboration with Dr. Gene Major (Loyola University) using a radioimmune precipitation (RIP) technique has shown the presence of T antigen in long term cultured cells. However, qualitatively, the amount of T antigen in the cultured owl monkey tumor cells appears to be far less than that in JC transformed hamster tumor cells where viral antigen is readily detectable both by immunofluorescence and by the RIP technique.

Growth of the JC virus. The virus has been shown to infect human kidney and human amnion cells in a limited fashion with production of T antigen. Occasionally the virus has been reported by others to go through to production of infectious virus in these cells but passage of the virus has been difficult. To date we have not been successful at adapting our virus to productive growth in these cells.

We have cloned JC virus in the E. coli plasmid pBR 322 and have purified the pBR/JCV recombinant DNA. We have also cloned the closely related BK virus by a similar procedure. Molecular "probes" have been successfully prepared by "nick" translation of the cloned purified JCV DNA and have been shown to differentiate JCV from related papovaviruses.

Little is known about the immunologic response to JC virus or the mechanism of immune control. It is apparent that immunologic factors play an important role in disease expression. To date the analysis of the immune response has been limited to the detection of T antibody (non-specific for JC as it reacts with BK and SV-40 T antigen) and to an antibody detected by hemagglutination (some cross reactivity with BK virus). Further progress in defining the immune response to JCV has been impeded by the lack of specific antigens.

With the limited quantities of virus available, we have developed an enzyme-linked immunosorbent assay (ELISA) for detecting antibody to virus coated antigen. This assay is far more sensitive than existing procedures and is specific for JC and will allow for distinction with BK and SV-40 virion antigens.

Primate Brain Tumor Model - The development of the primate model for JC induced glioma production is also of interest and will continue under study. We have developed radioactive hybridization probes to detect JC virus in the tumors as well as the tumor cells in culture. We have investigated the condition of the genome in the tumor cells by hybridization of the cloned viral DNA probes to restriction endonuclease-digested tumor cell DNA. We have found that the tumor cell DNA has integrated JC viral sequences which are not present in non-tumor tissue from the same animal.

In conjunction with the Surgical Neurology Branch (Dr. Paul Kornblith, NINCDS) we plan to pursue studies regarding demonstration of a glioma specific cell surface antigen which might cross react with the human glioma surface antigen.



Monoclonal antibody to this antigen might facilitate early detection of this antigen in serum or CSF. The animal brain tumor model has been tested with the CAT scan, and we found that tumors approximately 1 cm in size could be detected by the scan.

c. Chronic Neurologic Diseases - The immune response of MS patients to viral (V) associated and brain cell associated (CNS) antigens remains under active study. The patients are studied for their antibody responses to myelin, basic protein and axolemma and such viruses as measles, rubella, CMV, EBV, HSV-1, coronaviruses and Vaccinia virus. Cellular immunity to these same antigens are also studied using lymphocyte stimulation assay. The immunoregulatory T-cell responses to these antigens are also tested.

We have detected interferon in 21/50 (43%) of active patients (during exacerbation) in cerebrospinal fluid and in 8/57 (14%) of non-MS controls with other neurological diseases. In contrast, no difference between patients and controls was found in serum levels of interferon.

Immune complexes were demonstrated to be significantly elevated in 32/50 (64%) of MS patients in exacerbation using the radioimmune Raji cell assay. No differences from controls were found using serum samples from patients in exacerbation or in stable condition.

We have examined a large number of patients for nonspecific and antigen related induction of in vitro T-suppressor cell activity. We have reported that normal matched pal controls and stable MS patients have similar levels of suppressor cell activity induced by Concanavalin A. This study demonstrated that the reported depression in T-cell suppressor function is not a genetic or persistent abnormality which occurs throughout the disease but may be more temporally related to the activation phase of the disease. In our recent studies some MS patients in the acute phase of disease have a depressed response by the T-suppressor cell population in response to induction by Concanavalin A in contrast to normal controls and patients with other neurological diseases.

However, many of our MS patients undergoing active exacerbations have a normal functional T-suppressor cell response which falls within the range of activity expressed by nondiseased individuals.

Antigen specific suppressor cell activity was induced in response to measles virus, rubella virus and occasionally Epstein-Barr virus in MS patients in a higher frequency than controls. Herpes simplex virus (1), cytomegalovirus and influenza (A) viruses were never shown to induce suppressor cell activity. Although antibody levels were frequently elevated in MS patients to these same viruses (measles, rubella, EBV) no direct correlation could be found between the induction of suppressor cell response and the antibody titer.

A loss of normal T-suppressor cell response was shown in some stable MS patients and more frequently in patients with active disease in response to brain related antigens (myelin and axolemma). Occasionally, positive cellular proliferative reactions were demonstrated in MS patients' lymphocytes in the presence of these brain related antigens when the suppressor cell response was depleted. The significance of this form of cell mediated autoimmune reaction is currently under study.

Proposed Course of the Project: We propose to:

- a. Continue studies of the relationship of EBV to chronic and acute neurologic diseases;
- b. Continue studies on the relationship of JC virus to human disease and to determine mechanisms of immune control and to continue studies of pathogenesis of JC virus in owl monkeys;
- c. Continue studies on role of immunoregulatory response in patients with MS;
- d. Pursue studies on the mechanisms of ADLC and NK activity and the role of T<sub>γ</sub> cells in this response and the influence of immune complexes on the expression of these functions.

Significance of Program to the Institute: Herpesviruses (HSV, CMV, EBV) and polyoma-viruses often establish persistent infections with neurological manifestations. Although neurologic complications of HSV and CMV infections are more well known, the clinical spectrum of disease associated with EBV or JC virus infections has not been completely defined. These studies are designed to examine the clinical spectrum of EBV-associated diseases of children, to describe the neurological involvement associated with these infections, and to determine whether this is an etiologic or opportunistic relationship between EBV infection and neurologic abnormalities. In addition, the studies of JC virus may provide useful information regarding the spectrum of diseases which this virus can induce.

The importance of delayed hypersensitivity in several viral infections has been well documented. However, the role of CMI in chronic viral diseases with neurological complications is less well studied. These studies should help define the role of delayed hypersensitivity in herpesvirus infections particularly EBV and JC virus which are followed by neurological dysfunction.

Determination of the role of immunoregulatory responses in contributing to pathogenesis in chronic neurologic diseases is of prime interest. Our studies in this area regarding patients with MS may help determine whether the disease is an autoimmune reaction and may help in understanding the role of infectious agents in this disease.

Publications:

1. Wallen, W.C., Houff, S.A., Iivanainen, M., Calabrese, V.P. and DeVries, G.H.: Suppressor cell activity in multiple sclerosis. Neurology 31:668-674, 1981.
2. Rentier, B. and Wallen, W.C.: Scanning and transmission electron microscopy study of antibody-dependent lymphocyte mediated cytotoxicity on measles virus-infected cells. Infection and Immunity 30:303-315, 1981.
3. Madden, D.L., Wallen, W.C., Holmes, K., Castellano, G., Houff, S., Shekarchi, I., Leinikki, P. and Sever, J.L.: Occurrence of coronavirus antibody in sera from multiple sclerosis patients and matched controls. Archives of Neurology (In press) 1981.

4. Madden, D.L., Wallen, W.C., Houff, S., Shekarchi, I., Leinikki, P., Castellano, G.A., Holmes, K. and Sever, J.L.: Humoral and cellular immune responses of multiple sclerosis patients. In Boese, A. (Ed.), Search for the Cause of Multiple Sclerosis and Other Chronic Diseases of the Central Nervous System, from the First International Symposium of Hertie Foundation in Frankfurt/Main, September 1979, pp. 442-453, 1980.

5. Iivanainen, M., Wallen, W., Leon, M.E., Keski-Oja, J., Calabrese, V., Krasny, M., Waybright, E., Selhorst, J., Harbison, J., Madden, D., and Sever, J.: Micromethod for detection of oligoclonal IgG in unconcentrated cerebrospinal fluid by polyacrylamide gel electrophoresis. Archives of Neurology (In press) 1981.

6. Calabrese, V., Wallen, W.C., Castellano, G., Ward, L., Anderson, G. and DeVries, G.: Enzyme-linked immunosorbent assay (ELISA) for antibodies to human myelin and axolemma-enriched fraction. Neuroscience Letters 21:189-195, 1980.

7. Madden, D.L., Wallen, W.C., Houff, S.A., Shekarchi, I.C., Leinikki, P.O., Castellano, G.A. and Sever, J.L.: Measles and canine distemper antibody. Presence in sera from patients with multiple sclerosis and matched control subjects. Arch. Neurol. 38:13-15, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  <div style="text-align: center; font-weight: bold;">Z01-NS-01984-10-ID</div>																								
PERIOD COVERED <div style="text-align: center; font-weight: bold;">October 1, 1980 to September 30, 1981</div>																										
TITLE OF PROJECT (80 characters or less) <div style="text-align: center; font-weight: bold;">Maternal Infection and Pregnancy Outcome</div>																										
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																										
<table style="width: 100%; border: none;"> <tr> <td style="width: 10%; vertical-align: top;"><b>PI:</b></td> <td style="width: 30%;">William C. Wallen</td> <td style="width: 30%;">Senior Staff Fellow</td> <td style="width: 30%;">IDB, IRP, NINCDS</td> </tr> <tr> <td style="vertical-align: top;"><b>Other:</b></td> <td>John L. Sever</td> <td>Chief</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td></td> <td>David L. Madden</td> <td>Veterinary Director</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td></td> <td>William T. London</td> <td>Veterinary Director</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td></td> <td>John H. Grossman</td> <td>Guest Worker</td> <td>IDB, IRP, NINCDS</td> </tr> <tr> <td></td> <td>Frank J. West</td> <td>Biological Lab Technician</td> <td>IDB, IRP, NINCDS</td> </tr> </table>			<b>PI:</b>	William C. Wallen	Senior Staff Fellow	IDB, IRP, NINCDS	<b>Other:</b>	John L. Sever	Chief	IDB, IRP, NINCDS		David L. Madden	Veterinary Director	IDB, IRP, NINCDS		William T. London	Veterinary Director	IDB, IRP, NINCDS		John H. Grossman	Guest Worker	IDB, IRP, NINCDS		Frank J. West	Biological Lab Technician	IDB, IRP, NINCDS
<b>PI:</b>	William C. Wallen	Senior Staff Fellow	IDB, IRP, NINCDS																							
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	John H. Grossman	Guest Worker	IDB, IRP, NINCDS																							
	Frank J. West	Biological Lab Technician	IDB, IRP, NINCDS																							
COOPERATING UNITS (if any)  <div style="text-align: center; font-weight: bold;">George Washington University Medical School, Washington, D.C.</div>																										
LAB/BRANCH <div style="text-align: center; font-weight: bold;">Infectious Diseases Branch</div>																										
SECTION <div style="text-align: center; font-weight: bold;">Neurovirology</div>																										
INSTITUTE AND LOCATION <div style="text-align: center; font-weight: bold;">NINCDS, NIH, Bethesda, Maryland 20205</div>																										
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SUMMARY OF WORK (200 words or less - underline keywords)  <p>           Pregnant women have normal immunocompetence in response to general mitogens and recall antigens. Specific <u>cellular immunity</u> to HSV-II antigen appears somewhat delayed in pregnant women with <u>recurrent infections</u> compared to <u>primary infections</u>. <u>Antibody levels</u> did not correlate with protection from recurrent infection. Antibody dependent lymphocyte cytotoxicity was more sensitive in detecting antibody to HSV-II than was conventional <u>immune hemagglutination assay</u>.         </p>																										
37 - IDB/IRP																										



### Project Description:

**Objectives:** Infectious diseases play a significant role in causing abnormal neurological development. We are in the process of studying several viruses to determine their role in the development of birth defects and neurological diseases. Sensitive virological and immunological techniques are currently being developed and applied to the investigation of the natural course of the disease induced by these viruses during pregnancy. Particular emphasis will be placed on Herpes simplex virus, type II (HSV-II) and cytomegalovirus (CMV) infections of pregnant women.

In addition we are studying the development of fetal immunity and its role in protection against viral infections which cause neonatal disease.

**Methods Employed:** HSV-II and CMV are routinely isolated employing such techniques as genome rescue by cocultivation of intact cells, cellular disruption to isolate intracellular infectious virus, as well as chemical or mitogenic activation of latent genome from infected tissues, biopsies or leukocyte populations.

Several parameters of humoral and cell-mediated immunity (CMI) are routinely employed to determine a comprehensive immunologic response pattern to a viral infection. We are currently employing indirect hemagglutination (IHA), virus neutralization, fluorescent antibody assays, ELISA and the antibody dependent lymphocyte cytotoxicity (ADLC) assay for antibody measurement to these viral antigens.

Assessment of cellular immunity includes lymphocyte stimulation assay, leukocyte migration inhibition and immune interferon assays. The distribution of lymphocyte subpopulations in pregnant women and newborns is determined employing rosetting or immunofluorescent assays for quantitation of peripheral blood T- and B-cells.

General immunocompetence of host lymphocytes was performed employing the lymphocyte stimulation assay to evaluate the proliferative response to general mitogens (phytohemagglutinin (PHA), Concanavalin A (Con A) staphylococcus lysate antigens). In selected cases, the ability of the individual's lymphocytes to participate in the ADLC assay was measured with both autologous and known positive sera.

**Major Findings:** Studies regarding immunity to HSV-II infection during pregnancy have revealed:

1. Symptomatic pregnant women develop the same level of cellular immunity (lymphocyte stimulation) and humoral immunity (Indirect Hemagglutination, ADLC) to HSV II and in the same frequency as symptomatic nonpregnant women;
2. Pregnancy does not compromise general immunocompetence regarding these parameters of immunity;
3. There appears to be a delay in development of CMI in recurrent infections compared to primary infections;



4. During active shedding of virus, CMI is active only in about 40% of the cases, suggesting some inhibitory factor;
5. In primary infections, ADLC response is delayed compared to IHA antibody response.

A model for neonatal CMV central nervous system infection was developed utilizing fetal rhesus monkeys infected with a natural rhesus CMV isolate. We have produced a CNS infection by direct inoculation of virus at 50 and 80 days of gestation. Virus was readily isolated from brain tissue after normal delivery. Maternal antibody levels were elevated during fetal infection suggesting placental transfer of virus to the mother. This model will be further developed virologically and immunologically to examine the pathogenesis of CMV infection during fetal development and persistent CNS infection.

Significance of the Program to the Institute: These studies regarding the natural course of HSV-dII and CMV infections in pregnant women and in newborns may help to determine the pathogenesis of these latent, persistent viruses. In addition, these studies may delineate mechanisms of immunological control of these viruses under normal conditions. It would be of importance to determine the contribution that immunity or immune deficits play in development of viral latency or persistence and to the subsequent neurological dysfunction that may occur later in life as a result of infections with these viruses.

Proposed Course of the Project: Studies regarding the natural history of HSV-I and II and CMV in pregnant women and its consequences to newborns will continue on a longitudinal basis. Immunological studies will be used to determine:

- 1) the role immunity plays in control of disease;
- 2) tests for prognostic evaluation using various viral-related antigens; and 3) the mechanisms of protection of the newborn and the contribution of immunity to latency in the newborn.

Nonhuman primates will be employed for more definitive studies regarding the natural course of CMV and Herpes simplex virus, as well as to study the parameters of immunity during fetal development and postnatally.

#### Publications:

1. Kumar, A., Selim, M.S., Madden, D.L., Wallen, W.C., Vasquez, H.H. and Nankervis, G.A.: Humoral and cell-mediated immune responses to herpesvirus antigens in patients with cervical carcinoma. Gynecologic Oncology 10:18-25, 1980.
2. Grossman, J.H. III., Wallen, W.C., and Sever, J.L.: The management of genital Herpes simplex virus infections during pregnancy. Ob/Gyn. (in press) 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  <div style="text-align: center; font-weight: bold;">Z01-NS-00972-10-ID</div>
PERIOD COVERED <div style="text-align: center; font-weight: bold;">October 1, 1980 to September 30, 1981</div>		
TITLE OF PROJECT (80 characters or less) <div style="text-align: center; font-weight: bold;">Role of Viruses and Other Microorganisms in the Perinatal Period of Experimental Animals.</div>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
<b>PI:</b>  William T. London James S. Harper, III	Veterinary Director Veterinary Medical Officer	IDB,IRP,NINCDS IDB,IRP,NINCDS
<b>Other:</b> John L. Sever William C. Wallen Blanche L. Curfman Robert L. Brown Frank J. West	Chief Senior Staff Fellow Biologist Biological Lab Technician Biological Lab Technician	IDB,IRP,NINCDS IDB,IRP,NINCDS IDB,IRP,NINCDS IDB,IRP,NINCDS IDB,IRP,NINCDS
COOPERATING UNITS (if any) University of Pittsburgh Presbyterian Hospital, Department of Neuropathology Pittsburgh, Pennsylvania Meloy Laboratories, Inc., Springfield, Virginia		
LAB/BRANCH Infectious Diseases Branch		
SECTION Experimental Pathology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
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CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Venezuelan Equine Encephalitis Vaccine strain TC-83 (VEE) reproducibly produces teratogenic effects in CNS tissue of fetal rhesus monkeys when inoculated intracerebrally into 100-day fetuses. This presents an opportunity to study the pathogenesis of viral-induced micrencephaly, hydrocephalus, porencephaly and cataracts.  Simian cytomegalovirus in rhesus monkeys (Rh-CMV): We have produced congenital infection and disease in rhesus monkey fetuses after intrauterine inoculation of Rh-CMV in seropositive mothers.  Western Equine Encephalitis (WEE): Fetal rhesus monkeys were inoculated intracerebrally with an experimental vaccine strain of WEE virus. All animals developed micrencephaly. Twelve of 16 monkeys developed ex-vacuo hydrocephalus. All virus-inoculated fetuses developed WEE virus antibody. Virus could not be recovered at the time of delivery.  Congenital Toxoplasmosis: In order to develop a nonhuman primate model for congenital toxoplasmosis, we screened several species of nonhuman primates and concluded that <u>patas Erythrocebus patas</u> is the monkey most suitable for studies of acquired toxoplasmosis. These animals were readily infected by oral administration of toxoplasma cysts.		

Project Description:

Objectives: To study the role of viruses and other microorganisms in the perinatal period, the infection of gravid and non-gravid animals of several different species by parenteral routes with various viruses and other microorganisms to determine the effects of these agents on the animals and their fetal tissues.

Attempt to recover inoculated agents from the various animals and fetal tissues and the correlation of these re-isolations with gestational age at inoculation and dosage given. Relate these findings with gross and histopathological findings. Correlate all of this information with serological findings.

Methods Employed: An investigation of the role of viruses and other microorganisms in the perinatal period by the continual use of experimental animals, tissue culture techniques, histopathological studies and serological testing. Pregnant monkeys were inoculated by various routes and times of gestation with viruses and held in isolation chambers throughout the experiment. The animals were observed and monitored by serum samples, spinal fluid, throat swabs and tissue biopsy for evidence of disease and/or effects on fetal tissue. Pregnant animals were delivered by cesarean section so all products of conception could be saved.

Major Findings:Venezuelan Equine Encephalitis:

Rhesus monkey fetuses have been inoculated intracerebrally with VEE vaccine virus at 100 days of gestation.

Fetuses are delivered sequentially every 10 days postinoculation until term (160 days). We have found that after 10 days the virus can no longer be isolated from the fetal tissues. However, the fetuses show lesions in the CNS. The lesions become progressively more severe with time until at full term the animal has severe porencephaly, hydrocephaly and micrencephaly.

All fetuses delivered after 140 days gestation show signs of bilateral cataracts. At term the cataracts are fully developed in every inoculated animal.

Rhesus Monkey Cytomegalovirus (Rh CMV):

We have been able to produce congenital infection and disease in fetal Rhesus monkeys following intrauterine inoculation with Rh-CMV in sera-positive mothers. At 80 days gestation, five Rhesus fetuses inoculated intracerebrally with Rh CMV became infected and virus was isolated from their tissues. At birth, four of these animals showed severe hydrocephalus. Five more pregnant animals were inoculated intraamniotically at 50 days gestation. Their fetuses became infected as demonstrated by virus isolation from fetal tissues. However, only two of these newborn had hydrocephalus. Control animals did not develop congenital disease nor did CMV antibody titers rise in the mothers as it did in the mothers bearing inoculated fetuses. Rh CMV is a common viral infection of feral rhesus monkeys.

About 80 - 90% of adult animals have had the disease and many are persistent excretors of the virus. This is so similar to human CMV infections that we believe the monkey model would provide a useful tool to study this important human disease and its effects during pregnancy.

#### Western Equine Encephalitis:

Fetal rhesus monkeys were given intracerebral inoculations with the WEE vaccine at 100 days gestation age. These inoculations resulted in micrencephaly and hydrocephalus without aqueductal stenosis. All fetal monkeys developed micrencephaly; 12 of 16 monkeys developed hydrocephalus of the ex vacuo type.

Mechanisms by which WEE virus-induced hydrocephalus might occur include the arrest of brain parenchymal development, necrosis of brain tissue, reduced reabsorption of cerebrospinal fluid (CSF) or increased production of CSF. At the time of necropsy, the fetal monkeys exhibited no evidence of increased cerebrospinal fluid in the extraventricular system. The brain weights of the monkeys with hydrocephalus were significantly lower than those of control animals. Therefore, WEE virus produced the effect of micrencephaly and hydrocephaly by arresting brain parenchymal development in such a way as to cause hydrocephalus ex vacuo.

#### Congenital Toxoplasmosis:

In order to develop a nonhuman primate model for congenital toxoplasmosis we have screened several species of nonhuman primates and found that the patas (Erythrocebus patas) is the monkey most suitable for studies of acquired toxoplasmosis. They were readily infected by oral administration of toxoplasma cysts. Viable organisms were isolated from biopsied lymph nodes 30 days after inoculation. In this same study we found that squirrel monkeys (Saimiri sciureus) are highly susceptible to toxoplasmosis; all inoculated animals died in seven to nine days. Rhesus monkeys (Macaca mulatta) are essentially resistant to Toxoplasma gondii.

#### Significance of the Program to the Institute:

Research for animal models for human diseases known or suspected to cause malformations of the central nervous system should provide an insight into the pathogenesis of these anomalies. Epidemiological studies have shown that there are several viral teratogens in the human populations. These could be more thoroughly studied in animal models. Environmental agents alone or in combination with infectious agents may play a role in the development of certain types of congenital malformations. Animal models would certainly be useful in the study of these conditions.

#### Proposed Course of the Project

##### VEE STUDY:

All work on VEE study is completed and manuscript will be submitted for publication in near future.



Rhesus CMV Model:

Future work will focus on the completion of the neuropathological studies on the above animals and publication of results. Additional animals will be inoculated and the CMV-infected neonates, with and without congenital anomalies, will be compared by humoral and cellular immune tests and for presence of virus. These baby monkeys will be observed for one year for the following suspected, but not proven, CMV-related anomalies: Eighth nerve or cochlear deafness, infantile spasms with hypsarrhythmia and chorioretinitis and retinal necrosis and calcification.

Western Equine Encephalitis:

The results have been published and no further work will be done in this area.

Congenital Toxoplasmosis:

Future work will focus on establishing a congenital infection in patas monkeys. We will inoculate pregnant patas monkeys with tissue culture grown trophozoites using the intra-amniotic route. This will allow us to study the teratogenic effect of the organism on the patas monkey fetus. From previous experience with other agents inoculated into the gravid uterus, several animals will be needed to accurately establish the optimum number of organisms to be inoculated. After establishing infection by intraamniotic inoculation, we propose to infect another group of pregnant patas monkeys orally using brains from chronically infected mice. The pregnant animals will be infected late in pregnancy (120 days) as observations in humans suggest that the placenta is more easily crossed by the parasite at the end of pregnancy.

Publications:

W.T. London, Levitt, N.H., Altshuler, G., Curfman, B.L., Kent, S.G., Palmer, A.E., Sever, J.L. and Houff, S.A.: Teratological effects of western equine encephalitis virus on the fetal nervous system of Macaca mulatta. Teratology. Accepted for publication 05/12/81. In Press.



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  <div style="text-align: center; font-weight: bold;">Z01-NS-01986-10-ID</div>
PERIOD COVERED <b>October 1, 1980 to September 30, 1981</b>		
TITLE OF PROJECT (80 characters or less) <b>Inoculation of Animals with Tissue Culture Grown Materials          from Patients with Chronic Neurological Diseases</b>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
<b>PI:</b>  <b>Other:</b>	William T. London  Sidney A. Houff John L. Sever Blanche L. Curfman Robert L. Brown	Veterinary Director  Clinical Associate Chief Biologist Biological Lab Technician
		IDB, IRP, NINCDS  IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS
COOPERATING UNITS (if any) <b>Meloy Laboratories, Springfield, Virginia          Microbiological Associates, Bethesda, Maryland</b>		
LAB/BRANCH <b>Infectious Diseases Branch</b>		
SECTION <b>Experimental Pathology</b>		
INSTITUTE AND LOCATION <b>NINCDS, NIH, Bethesda, Maryland 20205</b>		
TOTAL MANYEARS: <div style="text-align: center; font-weight: bold;">1.4</div>	PROFESSIONAL: <div style="text-align: center; font-weight: bold;">0.4</div>	OTHER: <div style="text-align: center; font-weight: bold;">1.0</div>
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS         </div> <div> <input type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input checked="" type="checkbox"/> (c) NEITHER         </div> </div> <div style="margin-top: 5px;"> <input type="checkbox"/> (a1) MINORS    <input type="checkbox"/> (a2) INTERVIEWS       </div>		
SUMMARY OF WORK (200 words or less - underline keywords) <p>Young cynomolgus monkeys <u>Macaca fascicularis</u> were inoculated intracerebrally with "Biken" strain of subacute sclerosing panencephalitis (SSPE) virus and monitored for clinical signs of disease. This is a long term project and signs of disease may not appear for 30 months, as was the case with one animal previously inoculated with this strain of SSPE virus.</p> <p>Attempts to rescue the "Delta Agent" from the dorsal root ganglion of experimentally infected patas monkeys <u>Erythrocebus patas</u> were unsuccessful. This part of the study will be terminated.</p> <p>Simian Hemorrhagic Fever (SHF) Virus: A model for study of persistent infection. Four strains of SHF virus have been identified in patas monkeys <u>Erythrocebus patas</u>. Two produce an acute infection in patas monkeys with the infected animal eliciting a high titer antibody response and complete recovery. The other two strains of SHF virus produce a persistent infection with low levels of circulating antibody.</p>		

Project Description:

**Objectives:** Develop nonhuman primate models for the study of chronic neurological diseases (other than spongiform encephalopathies).

**Methods Employed:** Measles antibody negative cynomolgus monkeys were inoculated intracerebrally with subacute sclerosing panencephalitis (SSPE) "Biken" strain virus and monitored for infection and clinical signs.

Laboratory reared patas monkeys that are antibody negative to Delta herpes virus inoculated with "Delta" herpes virus to produce a latent infection.

Major Findings:SSPE Studies:

Cynomolgus monkeys with high measles antibodies have been inoculated with "Biken" strain of SSPE virus. This virus has produced clinical neurological signs in a cynomolgus monkey 30 months post inoculation. We are now monitoring our newly inoculated monkeys.

"Delta Agent" Studies:

Attempts to rescue the latent "Delta agent" a varicella-like virus from the dorsal root ganglion of patas monkeys has been unsuccessful.

Simian Hemorrhagic Fever Studies:

The SHF syndrome in patas monkeys offers an excellent opportunity to study a persistent viral infection in an animal model that is large enough to allow repeated daily samplings to monitor the infection process. We have identified four strains of SHF virus. Two represent acute strains that produce acute infection (one more severe) in patas monkeys. After 14 -21 days, the animals eliminate the virus and develop high levels of circulating antibody. Two additional strains have been identified as persistent. That is in patas monkeys, the persistent viral strains are not cleared and the infected animals do not develop high titers of antibody. One of the persistent strains can not be grown to detectable levels in macrophage cultures. The other three strains are readily grown and detected in vitro.

**Significance of the Program to the Institute:** Chronic neurological disease represents by far the major portion of the practice of neurology in the United States. Primate models may give answers to the pathogenesis of these diseases. Pathogenic principles derived from these models may then be applied to other chronic neurological diseases.

Proposed Course of the Project:

a. SSPE project: We will continue monitoring the animals inoculated intracerebrally with "Biken" strain of SSPE virus for signs of disease. Animals exhibiting seizures will be examined using electroencephalography, computed tomography, cellular and humoral immunity and oligoclonal banding.

b. Latent "Delta Agent" Infection: We have several animals that are latent carriers of "Delta Agent" varicella-like virus. We are investigating the immune mechanisms related to this virus reactivation by direct immunosuppression with chemotherapy and by supra-infection with another virus.

c. This SHF animal model would be ideal for studies of chemotherapy of persistent viral infection. A small group of persistently infected patas monkeys will be treated with new antiviral drugs to determine the efficacy of the drug and the physiological effects in nonhuman primates.

Publications: None

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  <div style="text-align: center; font-weight: bold;">Z01-NS-02136-07-ID</div>
PERIOD COVERED <b>October 1, 1980 to September 30, 1981</b>		
TITLE OF PROJECT (80 characters or less) <b>Control of Acute Infectious Diseases in Experimental Animals Using Biologicals and Chemotherapeutic Agents</b>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	William T. London Amos E. Palmer James S. Harper III	Veterinary Director Veterinary Director Veterinary Medical Officer  IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS
Other:	John L. Sever William C. Wallen John W. Larsen Blanche L. Curfman Robert L. Brown Robert M. Chanock	Medical Director, Chief Senior Staff Fellow Guest Worker Biologist Biological Lab Technician Medical Director  IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS LID, IRP, NIAID
COOPERATING UNITS (if any) Meloy Laboratories, Inc., Springfield, Virginia; LID, IRP, NIAID; Microbiological Associates, Bethesda, Maryland; Div. of Pathology, New England Regional Primate Research Center, Southborough, Mass.		
LAB/BRANCH Infectious Diseases Branch		
SECTION Experimental Pathology		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS:	PROFESSIONAL:	OTHER:
3.4	1.4	2.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINGRS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) Group B Streptococcus type III (GBS) studies - Antepartum penicillin therapy in pregnant rhesus monkeys infected intraamniotically with GBS significantly reduces neonatal mortality and associated morbidity.  Herpes encephalitis - We have had difficulty in reproducing our results in the rhesus <u>Macaca mulatta</u> and cynomolgus <u>Macaca fascicularis</u> monkeys. We have obtained a new "low passage" strain that is extremely virulent in hooded rats. We will inoculate monkeys with this virus.  Varicella-like "Delta Agent" encephalitis - The Delta herpes virus (DHV) will produce encephalitis and pneumonia in young DHV antibody negative patas monkeys <u>Erythrocebus patas</u> . This model can be used to study pathogenesis, diagnosis and treatment of human varicella encephalitis.		



## Project Description:

Objectives: To study prophylactic and therapeutic agents for the prevention and control of infectious diseases. The testing of candidate vaccines as to their immunogenicity, communicability and safety in experimental animals.

Methods Employed: New chemotherapeutic agents which show promise are studied in appropriate experimental animals. The animals are inoculated with a known infectious agent, then a therapeutic regimen is started, using the test drug. Additional animals are prophylactically treated with the drug, then challenged with the infectious agent.

Biological agents are tested for their ability to protect animals against naturally occurring and experimentally produced infectious diseases. Newly developed vaccines will be tested in susceptible experimental animals. Vaccinated animals will be exposed to susceptible sentinel animals to determine communicability. Vaccinated animals will be challenged at appropriate times to determine the immunogenicity of the vaccine.

## Major Findings:

Group B Streptococcus type III (GBS) studies: Antepartum treatment of GBS infection was done in the rhesus monkey model. The treated animals received a single simultaneous intravenous and intramuscular injection of penicillin following intra-amniotic inoculation of GBS. The penicillin group had a significantly lower neonatal mortality (one of nine) than controls (six of ten). Both groups of rhesus mothers developed a significant increase in antibody titer to GBS; however, the antibody response was of lesser magnitude in the penicillin treated group.

Herpes simplex encephalitis (HSE): We have been able to produce HSE using two species of monkeys (Macaca fascicularis and Macaca mulatta). Only animals which are antibody negative to herpesviruses have been used. This is important since antibody to Herpes simiae virus, a common infection in macaques, will neutralize Herpes simplex virus (HSV). We have had trouble with the strain of HSV 1 we were using and could not reproduce the HSE in our monkeys. Recently, we obtained a strain of HSV that has been extremely virulent in hooded rats. Using this strain of HSV, we are now inoculating monkeys to reproduce HSE.

Varicella-like "Delta agent" encephalitis: Patas monkeys Erythrocebus patas inoculated intracerebrally with Delta herpesvirus (DHV) develop clinical signs of pneumonia and encephalitis. Intratracheal or intravascular inoculations of DHV produce pneumonia but not encephalitis. Prominent microscopic lesions include diffuse interstitial pneumonia and focal hemorrhagic encephalitis. Typical herpesvirus intranuclear inclusion bodies were noted in lung parenchymal cells and in glial cells of the brain. DHV was isolated from peripheral blood leukocytes, spleen, lymph nodes, lungs and from various sites in the peripheral and control nervous system.

Significance of the Program to the Institute: Experimental animal studies permit the study of human diseases, their prevention and treatment with chemotherapeutic agents and biological products. Such studies provide information of efficacy, safety and side effects of these products. Information gained from experimental animal studies provides the bridge to the implementation of clinical studies in man.



Proposed Course of the Project:

Group B Streptococcus type III (GBS) studies: Future work will focus on the use of modified immune serum globulin (human) used as part of the therapy in the critically ill newborn infant. This material has shown promise in rats and mice. The rhesus monkey model will be used to determine the efficacy of this product in protecting a nonhuman primate. We will investigate the possibility of GBS antibody acquired naturally or by vaccination protection of infants from challenge with GBS 24 hours prior to delivery.

Future work will focus on pathogenesis, diagnosis and treatment of HSE. Both species of cynomolgus Macaca fascicularis and rhesus macaca mulatta monkeys will be inoculated intracranially and virus replication and spread within the nervous system, immunological controlling factors (i.e. cellular, humoral immunity) and the effects of steroids on development of lesions will be investigated.

Cynomolgus monkeys will be inoculated on the abraded cornea of the eye. In other models, mice and rabbits develop persistent viral infection of the trigeminal ganglion but do not develop HSE in a manner analogous to humans. We hope to induce persistent HSV infection of the trigeminal ganglion and then challenge the animal with stress (i.e. steroids and epinephrine), to produce fatal encephalitis. The development of this model will provide an experimental infection which possibly parallels the pathogenesis which occurs in humans. Furthermore, the persistence in the trigeminal ganglion will allow further investigation of the mechanism of this persistence and the mode of control of such infections.

Varicella-like "Delta agent" encephalitis: This virus becomes latent in patas Erythrocebus patas monkeys following a primary infection as does varicella zoster virus (VZV) in humans. We have evidence that we can activate latent DHV by superinfection of the monkey with another virus. This would provide a model for study of the reactivation of latent viruses. Monkeys that are latent carriers of DHV will be chemically immunosuppressed to determine if this will activate their latent infection.

Publications:

Murphy, B.R., Sly, D.L., Hosier, N.T., London, W.T. and Chanock, R.M.: Evaluation of three strains of influenza A virus in humans and in owl, cebus and squirrel monkeys. Infection and Immunity, 28(3): 688-691, 1980.

Larsen, J.W., London, W.T., Palmer, A.E., Curfman, B.L. and Bronsteen, R.A.: Penicillin treatment for group B streptococcal meningitis in the rhesus monkey. Obstetrics and Gynecology, 57(3): 330-334, 1981.

Larsen, J.W., Jr., London, W.T., Baker, C.J., Curfman, B.L. and Sever, J.L.: Intra-amniotic infection due to group B streptococcus: Treatment and antibody response. Obstetrics and Gynecology. Accepted for publication 02/25/81. (In press).

Johnson, L.D., Palmer, A.E., King, N.W. and Hertig, A.T.: Vaginal adenosis in Cebus apella monkeys exposed to DES in utero. Obstetrics and Gynecology, 57(2): 629 - 635, 1981.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  <div style="text-align: center; font-weight: bold;">Z01-NS-02271-05-ID</div>
PERIOD COVERED <b>October 1, 1980 to September 30, 1981</b>		
TITLE OF PROJECT (80 characters or less)  <b>Papovaviruses in Non-human Primates</b>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
<b>PI:</b>  <b>Other:</b>	William T. London Sidney A. Houff  William C. Wallen John L. Sever Kenneth G. Rieth Giovanni Di Chiro Paul E. McKeever Robert L. Delapaz Blanche L. Curfman Robert L. Brown	Veterinary Director Clinical Associate  Senior Staff Fellow Chief Staff Radiologist Chief Medical Officer Medical Officer Biologist Biological Lab. Technician
IDB, IRP, NINCDS IDB, IRP, NINCDS  IDB, IRP, NINCDS IDB, IRP, NINCDS DR, CC NCT, SNB, NINCDS SNB, NINCDS NCT, SNB, NINCDS IDB, IRP, NINCDS IDB, IRP, NINCDS		
COOPERATING UNITS (if any) <b>University of Wisconsin Medical School, Departments of Medical Microbiology and Pathology, Madison, Wisconsin; DR, CC, NIH; SNB, NINCDS</b> <b>Meloy Laboratories, Inc., Springfield, Virginia</b>		
LAB/BRANCH <b>Infectious Diseases Branch</b>		
SECTION <b>Experimental Pathology</b>		
INSTITUTE AND LOCATION <b>NINCDS, NIH, Bethesda, Maryland 20205</b>		
TOTAL MANYEARS:  <div style="text-align: center; font-weight: bold;">1.4</div>	PROFESSIONAL:  <div style="text-align: center; font-weight: bold;">0.4</div>	OTHER:  <div style="text-align: center; font-weight: bold;">1.0</div>
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input checked="" type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  <b>Ninety two owl monkeys <u>Aotus trivirgatus</u> and 15 squirrel monkeys <u>Saimiri sciureus</u> that were inoculated in 1978 with JC virus, a human polyomavirus or control material have been maintained, monitored and removed from the study after 36 months (August, 1981). To date 13 owl monkeys and 3 squirrel monkeys have developed intracerebral neoplasms.</b>		

## Project Description:

**Objectives:** To study the pathogenesis of papovavirus induced tumors and disease in non-human primates.

Three serologically distinct papovaviruses have been isolated from humans. The JC and SV-40-PML strains have been isolated from patients with Progressive Multifocal Leukoencephalopathy (PML). BK virus has been isolated from the urine of renal transplant patients and at least one normal child.

Inoculation of JC, SV40-PML and BK viruses in hamsters, rabbits, rats, mice and bovines has resulted in tumors of various types. We have reported that JC virus inoculated by multiple routes produce astrocytomas in 2 of 4 adult owl monkeys.

We presently are confirming our original findings using a larger sample size, determining if the primary tumors from the original two owl monkeys can be transmitted to other owl monkeys, and investigating immune modulation of owl monkeys inoculated with JC virus to determine if this results in lesions resembling PML. Finally, is a closely related new world non-human primate, the squirrel monkey Saimiri sciureus more susceptible to JC infection than the owl monkey?

## Methods Employed:

Twenty adult feral Colombian owl monkeys (Aotus trivirgatus) were inoculated intravenously (IV) and intracerebrally (IC) with JC virus in attempts to confirm the original studies. Several owl monkeys were inoculated using a single variable route. This series included: a) intracerebral; b) intraperitoneal; c) intravenous; and d) inhalation. Another group of owl monkeys has been inoculated with primary tumor cells from the 2 original owl monkey gliomas.

Several owls have been immunosuppressed and then inoculated with JC virus attempting to produce PML-like lesions. Twenty-two monkeys were used as controls. A total of 92 animals were used in these studies.

Ten squirrel monkeys were inoculated with JC and 5 were inoculated with control material.

## Major Findings:

In the last year three additional owl monkeys inoculated intracerebrally with JC virus developed glioblastomas 20 months past inoculation. This brings the total to eight animals that have developed tumors following inoculation via multiple routes (intravenous, intracerebral and subcutaneous) or a single intracerebral inoculation.

Five of eight owl monkeys receiving immunosuppressive drugs several weeks before and after intracerebral JC virus inoculation developed neurological tumors. The mean time before clinical signs of tumors was 23 months. CNS tumor material taken from owl monkeys in a previous study was inoculated intracerebrally and to date two of six animals thus inoculated have developed tumors 15 months after inoculation. Three of ten intracerebrally inoculated squirrel monkeys have also developed glioblastomas.

Animals inoculated via other routes, i.e., intraperitoneal, intravenous or inhalation have not developed tumors and will be removed from the study in August, 1981 at 36 months post inoculation.

Significance of the Program to the Institute: Demyelinating diseases are a major cause of neurological disability in the United States. Multiple Sclerosis, Schilder's disease, Devic's syndrome, post-vaccination encephalomyelitis as well as PML are all illnesses characterized by loss of or defective myelin. The study of a known viral-induced demyelinating illness will hopefully give us the basic knowledge which is needed to understand the pathogenesis and etiology of the major white matter diseases of man. Brain tumors account for a relatively high proportion of all neurological disease. Gliomas are the most frequent tumors seen in man. JC virus has been shown to induce gliomas in primates. This is the first animal model of gliomas which will allow studies of pathogenesis, diagnostic techniques, and therapeutic trials applicable to human disease.

Proposed Course of the Project:

- a. Continue to process and examine tissues from the animals that have developed the tumors and write a complete report of our findings.
- b. Continue work on hybridization between JC virus DNA and DNA extracted from tumor cells will be done to delineate portions of JC virus DNA present in the tumor genome. These attempts may define which portions of JC virus are required for tumor induction in the nonhuman primate.
- c. Additional owl monkeys have been inoculated intracerebrally (24 virus and 6 controls). These animals will be monitored using monthly weights and quarterly hemograms as parameters. If weight loss is observed, computer tomography (CT) scans will be done immediately to locate possible tumors.
- d. As additional animals develop tumors, biopsies will be taken. Using in vitro cytotoxicity tests, the chemotherapy of choice will be determined and administered to the animals with tumors.

Publications: None



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02034-09 ID
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Electron Microscopic Studies of Viruses of the Nervous System and of Demyelination		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI: Monique Dubois-Dalcq Research Microbiologist IDB, IRP, NINCDS		
Other: Dr. B. Rentier Visiting Associate IDB, IRP, NINCDS Dr. B. Trapp Senior Staff Fellow IDB, IRP, NINCDS Anne Claysmith Biological Lab. Technician IDB, IRP, NINCDS Ray Rusten Biological Lab. Technician IDB, IRP, NINCDS Annik Baron PH.D. Student-Kroc Foundation IDB, IRP, NINCDS		
Collaborators: Dr. W. Bellini Senior Staff Fellow NIB, IRP, NINCDS Dr. R. Quarles Chief, Section on Myelin & Brain Development DMN, IRP, NINCDS Dr. K. Ramohan Clinical Associate NIB, IRP, NINCDS Dr. K. Holmes Associate Professor Department of Pathology USUHS, Bethesda		
COOPERATING UNITS (if any) Dr. M. Haspel, Dental Institute, NIH; LLNS, NINCDS: NIB, NINCDS. Dr. J. Griffin, Department of Neurology; Johns Hopkin University School of Medicine. Dr. Yan Duncan, Neurosciences Laboratory; Montreal General Hospital Quebec. Drs. Knobler, Oldstone and Lampert, Scripps Clinic and UCSD, La Jolla CA		
LAB/BRANCH Infectious Diseases Branch		
SECTION Electron Microscopy Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Md 20205		
TOTAL MANYEARS: 5.7	PROFESSIONAL: 2.7	OTHER: 3.0
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) In our structural studies of viral infections of nerve cells, we have demonstrated: (1) <u>In vivo</u> , the restricted tropism for neurons of a mutant of the wild JHM strain of a mouse coronavirus. (2) <u>In vitro</u> , a hepatotropic and a neurotoxic mutant of JHM strain have restricted tropism for neuron and the mutant shows abnormal assembly in CNS cells. (3) A wild measles strain can produce a selective infection of mouse neurons in the process of differentiation and the host cell is responsible for the establishment of virus persistence. Infected neurons appear to be unable to redistribute measles antibody complexes in their surface. In our studies on formation of myelin, we have demonstrated that: (1) P <sub>2</sub> , a peripheral nervous system protein, is also localized in rabbit oligodendroglial and CNS myelin. (2) The major peripheral nerve glycoprotein, Po, is localized on the cytoplasmic side of the Schwann cell plasma membrane, the outer mesaxons and the major dense line of compact myelin. The Golgi system and some cytoplasmic vesicles of Schwann cells also contain Po. (3) The myelin associated glycoprotein, MAG, is localized in the Schmidt-Lanterman incisures, the paranodal area and the outer mesaxons of actively myelinating Schwann cells. (4) <u>In vitro</u> , fibronectin promotes rat Schwann cell growth and motility.		



## Project Description:

Our approach to neurovirology is to study in vivo and in vitro the biology and structure of nerve cells and how these are affected by neurotropic viruses. The term "nerve cells," as used here, includes all nerve cell types in CNS and PNS. We are performing basic research with the hope of elucidating some mechanisms of viral and demyelinating diseases of the nervous system. We attempt to balance our research efforts and interests between neurobiology and neurovirology because viral expression in different nerve cell types can vary greatly with the properties and stage of maturation of these cells. We are studying myelin-forming cells and the emergence of myelin proteins during development in the PNS. Using immunocytochemistry, we also hope to analyze aspects of myelination and remyelination in the peripheral and central nervous system.

### A. Viral Infections of Nerve Cells

#### Objectives:

- (1) To analyze virus tropism and viral expression in developing and mature nerve cells.
- (2) To elucidate mechanisms of viral persistence in the nervous system.

#### Methods:

In Vivo: The nervous system of animals infected with various RNA viruses is studied with methods which allow light and EM localization of viral antigens in well preserved tissue section (vibratome, epoxy sections) in which nerve cell types and pathological events can be easily recognized.

In Vitro: The biology and maturation of various neurotropic viruses can now be easily studied in vitro with the development of (1) dissociated cultures of neurons and astrocytes, (2) dissociated cultures of myelin-forming cells, (3) CNS aggregates of differentiated cells. In dissociated CNS cultures, the virus input can be accurately controlled, specific antibodies and labeled precursors added to the medium come directly in contact with neurons and glial cell surfaces. Time-lapse VIM can be performed with phase or fluorescence microscopy and immunolabeling, and scanning EM can be done directly on the coverslip covered with cultured cells. CNS aggregated cultures have different advantages: large numbers of differentiated nerve cells are obtained, all nerve cell types within the CNS are present, sequential sampling and biochemical studies can be easily performed.

#### Major Findings:

In Vivo: A strong tropism for neurons, sometimes restricted to one major group of neurons, has been observed in three different virus-induced CNS diseases of the mouse. For example, a mutant (R<sub>1</sub>) of the rhabdovirus vesicular stomatitis virus (VSV) infects mostly anterior horn neurons where virus

maturation is followed by vacuolization and hind limb paralysis. Similarly, a measles neurotropic strain and the wild JHM strain of a mouse coronavirus (mouse hepatitis virus, MHV) both infect neurons and cause acute CNS disease. In these three viral CNS diseases, virus can be found in postsynaptic areas where it sometimes matures and spreads to presynaptic endings. In contrast, a temperature-sensitive mutant of the wild JHM strain of MHV (TS8) has restricted tropism for neurons and infects scattered oligodendrocytes, resulting in their progressive destruction and plaques of demyelination. The use of unembedded, non-frozen, chopped or vibratomed sections for immunoperoxidase labeling of viral antigens has been crucial for the observation of those results based on a tight correlation between light and electron microscopic observation.

In Vitro: We have studied various conditions in which persistent CNS infection with RNA viruses could be obtained in dissociated CNS cultures. In the case of measles virus, an important factor for viral persistence is a critical stage of maturation of the neurons, confirming the age-dependency of viral expression seen earlier in vivo. A wild measles strain can indeed produce a selective infection of a fraction of neurons in the process of differentiation, and this infection is characterized by a large accumulation of nucleocapsids and synthesis of all viral proteins with the hemagglutinin (HA) protein of measles virus covering the entire cell surface of the neuron body and neurites. However, there is a lack of incorporation of the nucleocapsids in the virions. No infectious virus is released except when infected neurons are cocultured with monkey kidney cells. The host cell is unable to complete viral maturation and is responsible for the establishment of persistence. The virus rescued by cocultivation is identical to the original wild virus.

This in vitro model is now used to study antibody modulation. Immune serum was obtained from a patient with SSPE and a hyperimmune serum against Edmonston measles was prepared in Balb/c mice. Both reacted with all measles polypeptides and showed reactivity to the surface of infected neurons. Using three monoclonal antibodies with known reactivity restricted to measles HA, the presence of HA on the cell surface was demonstrated. The altered mobility of HA was not due to the absence of a determinant recognized by any of these three monoclonal antibodies. The culture of infected neurons in the presence of these antibodies yielded interesting observations. Infected neurons continued to display bound Ig for as long as 21 days when monoclonal or polyclonal antibody was present in the medium either transiently (1 hour) or continuously. Antigenic patching, capping and shedding was not observed. No differences were observed if antibody was added on the first day of infection when viral antigens were not yet expressed on the cell surface or late in infection when antigenic expression was abundant. Whether these observations are relevant to infection in vivo is not clear. However, primary neurons in culture appear to be unable to redistribute immune complexes on their membranes.

The importance of the host cell in controlling virus tropism and expression can be optimally studied in an homologous system such as mouse neurotropic viruses in mouse dissociated CNS cells. The interaction of hepatotropic (A59)

and neurotropic (JHM) strains of the coronavirus MHV with murine CNS cells in vitro has been studied by EM and immunolabeling with antisera to the virion, its isolated glycoproteins [membrane E<sub>1</sub> and peplomeric E<sub>2</sub>] and glial fibrillary acidic protein (GFAP). As in vivo, wild type (WT) JHM produces acute cytopathic effects in a fraction of neurons and non-neuronal (NN) cells but infected neurons produce much less E<sub>2</sub> and fewer virions than the fused NN cells. In contrast, A59 and the TS8 mutant of JHM, which both produce chronic CNS disease, spare neurons and infect primarily astrocytes containing GFAP. These infected astrocytes are wrapped around uninfected neurons and progressively lose their fibrillar processes, but not GFAP. In NN cells, the intracellular budding of A59 and WT JHM virions is abundant and similar to that seen in other permissive cells. TS8 induces large inclusions of viral nucleocapsids and proliferation of smooth membrane, suggesting a defect in virus maturation. Both E<sub>1</sub> and E<sub>2</sub> are present in NN cells infected with all strains. Normal movement of the glycoproteins is observed with A59: E<sub>1</sub> remains in the perinuclear area while E<sub>2</sub> migrates throughout the cytoplasmic membranes. In contrast, E<sub>2</sub> is restricted to the perinuclear area of NN cells infected with the JHM strains. In conclusion: (1) two different MHV strains have restricted tropism for neurons; (2) specific properties of each virus strain may control tropism and block maturation; (3) the outcome of viral infection also depends on host cell factors.

In addition to our studies in rodent nerve cells, we are presently culturing dissociated and differentiated cells from various parts of the nervous system of human fetuses (as recently obtained by Dr. P. Kennedy in London). Cultures of dissociated sensory neurons were successfully obtained from each fetus using human placental serum containing medium. These human dorsal root ganglia neurons are very sensitive to mouse nerve growth factor. Long time-lapse video-recording of these cells (using video-intensification microscopy) reveal rapid growth of neurites which establish an extensive network within a few days. Sensory neurons adhere and grow as well on human plasma fibronectin as on rat tail collagen. At one week in vitro, neuron specific-enolase was detected by immunocytochemistry in virtually all cultured neurons and their processes. Scanning electron microscopy in stereo reveals interesting surface contacts between neurites and sensory neuron soma. Explant cultures of dorsal root ganglia could be maintained longer (4 to 5 weeks) than dissociated cultures (3 weeks). Human Schwann cells can also be cultured from fetal ganglia or nerves. Their motility behavior is different from that of "mature" Schwann cells.

Cultures of dissociated spinal cord from these human fetuses grow slowly in high serum medium. After one week, small bipolar or multipolar neurons, resembling spongioblasts, are identified and sometimes express neuron-specific enolase. The most numerous cells cultured from these human cord are astrocytes which strongly stain for glial fibrillary acidic protein. They either form clusters or are scattered among fibroblasts and show progressive differentiation during the next 3 to 4 weeks as in rodents.

### Significance of the Program to the Institute:

Measles in men, mouse hepatitis virus and VSV in animals, are all involved in acute and chronic infection of the CNS. Basic knowledge of the mechanism by which these viruses modify the cell membrane and, more specifically, the nerve cell membrane, will help us to understand the pathogenesis of disease. In addition, the mechanisms of viral persistence in the nervous system are still obscure. This year we have analyzed models in which either the nature of the virus (various strains of mouse hepatitis virus) or the host cell (maturational stage of neurons in measles infection) influences the outcome of viral infection. We have set up a system for culturing human nerve cells which differentiate in vitro. This will allow us to study interactions between human neurotropic viruses and human nerve cells and, therefore, improve our understanding of viral persistence in the nervous system in man.

### Proposed Course of the Project:

We are now culturing rat and mouse CNS aggregates in order to study viruses which have a tropism for oligodendrocytes. These cells are very rarely present in CNS dissociated cell cultures where no myelination has been detected so far. In contrast, CNS aggregated cultures contain oligodendrocytes and central myelin after three weeks in vitro. Therefore, virus tropism and replication in all CNS cell types can be studied in this system. Preliminary results suggest that the rhabdovirus VSV replicates well in CNS aggregated cultures. The molecular virology of such virus has been extensively studied in nondifferentiated cells but not in nerve cells. There is some evidence that rate of synthesis, transport and expression of viral proteins vary with cell types. As a model system, the synthesis and transport of the VSV glycoprotein could be studied in CNS aggregates by acrylamide gel electrophoresis and immunocytochemical methods. We have already prepared rabbit antiserum to the purified glycoprotein of VSV. More specifically, the presumed transport of this viral protein to the cell surface after glycosylation in the Golgi could be analyzed and visualized. It is important that such studies would be carried out in order to understand to which extent the molecular events of viral maturation are modified in nerve cells. We will also attempt to stain viral antigens in epoxy-embedded sections. This would allow us to test various antibodies against nerve cell markers and viral antigens on adjacent sections.

We also plan to develop cultures of human Schwann cells as well as neurons and glial cells from cerebellum and spinal cord of fetuses 12 to 20 weeks old with the hope to get in vitro maturation of all nerve cell types. We will infect these cells with various human neurotropic viruses.

One of the papova viruses, JC, can only replicate in differentiated human nerve cells and, therefore, our cultures can be used to isolate, characterize and grow JC virus from the brain of patients suffering from progressive multifocal leucoencephalopathy. In these cultures as well as in our rodent cultures, we will study the behavior and motility of various cell types by time-lapse VIM in phase as well as fluorescence microscopy. In this case



rhodamine-labeled probes, such as antibodies to various normal and viral cell surface proteins, will be used on living cells. We would also like to apply a newly described technique to the study of ultrastructural aspects of cultured nerve cells. There have been some recent spectacular developments of the fast-freezing and deep-etching techniques in the last couple of years. These have allowed new observations of cells frozen in the living state. Nobody has studied virus-infected cells and myelin with these techniques yet. We have recently acquired a copy of the fast-freezing machine developed by Reese and coworkers in the Institute and we are presently being assisted by Dr. Reese to make it functional. We think this technique allied with rotary shadowing will be a powerful tool to study the detailed interactions between intracellular components and virus-infected membranes as well as the relationship between myelin-forming cells and axons during demyelination and remyelination.

## B. Studies on Formation of Myelin

### Objectives:

To study (1) biochemical, morphological and immunocytochemical aspects of myelin formation and cellular differentiation both in vivo and in vitro and, (2) factors that trigger or modify the specific motility and function of myelin-forming cells, especially Schwann cells in the PNS.

### Methods:

In vivo: Immunocytochemical staining of myelin protein is performed with rabbit antibodies and with peroxidase-antiperoxidase complexes applied to epoxy sections previously treated with sodium ethoxide. Ultrastructural localization of Po protein in PNS myelin and Schwann cells is performed on teased fibers and vibratome sections with specific rabbit antibody and protein A-peroxidase.

In vitro: Rat Schwann cells (SC) are cultured from neonatal sciatic nerve following established techniques. Fibroblasts are eliminated by immune-mediated killing using monoclonal antibodies against Thy 1.1, a rat fibroblast surface protein absent on SC. Large quantities of pure SC are grown with mitogenic agents specific for SC (cholera toxin and a pituitary factor). Millions of subcultured SC are frozen away and used as needed. Primary and secondary SC are studied with phase, VIM, immunofluorescence, SEM and TEM. Mouse SC can also be purified from various strains of mice (except AKR). The starting culture system is adult mouse trigeminal ganglia in which neurons and fibroblasts can be eliminated by immune-mediated killing using monoclonal anti-Thy 1.2 antibodies. Mixed aggregates are obtained by mixing dissociated rat SC with a suspension of CNS cells or rat fetal brain. The ratio of SC/CNS cells is approximately 1/5 and both populations interact and reaggregate within hours on the rotary shaker. These aggregates are compared to aggregates made of CNS cells only.



Major Findings:

In vivo: The localization and emergence of various PNS myelin proteins are presently being studied by immunocytochemistry.

### 1. Localization of P<sub>2</sub> Protein in Rabbit Oligodendroglia and CNS Myelin

Antiserum directed against bovine peripheral nervous system P<sub>2</sub> protein has been used to localize P<sub>2</sub> protein in rabbit oligodendroglia and in rabbit central nervous system (CNS) myelin. Vibratome sections of rabbit spinal cord, brain stem and anterior commissure were stained immunocytochemically with P<sub>2</sub> antiserum by the peroxidase-antiperoxidase (PAP) method. P<sub>2</sub> antiserum stained myelin sheaths in all sections studied. Comparison of P<sub>2</sub> and myelin basic protein immunostained sections demonstrated that some myelin sheaths in the rabbit CNS were not stained by P<sub>2</sub> antiserum. Myelin sheaths not reacting with P<sub>2</sub> antiserum were found also in sections of rabbit sciatic nerves and they have been described in the rat peripheral nervous system (Trapp et al., PNAS, 76:3552-3556, 1979). Oligodendroglia in sections from 3-day-old rabbit brain stem were stained intensely by P<sub>2</sub> antiserum and stained processes extended from these perikarya to myelin sheaths. Antiserum directed against peripheral nervous system Po glycoprotein did not stain rabbit oligodendrocytes or rabbit CNS myelin. These results demonstrate that in the rabbit, P<sub>2</sub> protein is synthesized by oligodendroglia and is present in CNS myelin. Thus, P<sub>2</sub> protein cannot be considered exclusive to peripheral nerve myelin in all mammalian species.

### 2. Ultrastructural Localization of Po Protein in Developing Rat Schwann Cells

Po protein is a glycoprotein (MW 30,000) which represents over 50% of the total protein in peripheral nerve myelin. We have localized immunocytochemically Po protein in thin sections of actively myelinating rat peripheral nerves. Rats were perfused with 0.1% glutaraldehyde and 4.0% paraformaldehyde. The sciatic and trigeminal nerves were sectioned on a vibrating microtome, incubated successively with Po antiserum, protein-A-peroxidase and DAB, postfixed in 2% OsO<sub>4</sub>, dehydrated and embedded in epon. Penetration of the immunostaining reagents into the tissue was limited to 5-10 microns and the label was specific for myelinating Schwann cells. Analysis of thin sections revealed immunoprecipitate on the cytoplasmic side of Schwann cell plasma membranes, the outer mesaxon and loose non-compacted myelin. Compact myelin was not stained except in areas which were disrupted by sectioning on the vibratome. In these areas the major dense line of myelin was labeled. Po labelling was also found on the cytoplasmic side of Golgi cisterns and cytoplasmic vesicles. Occasionally, these labelled vesicles appeared to be fused with the Schwann cell plasma membrane. Nonmyelinating Schwann cells, neuronal cell bodies in trigeminal ganglion sections, axons, fibroblasts and connective tissue were not stained by Po antiserum. In sections incubated with preimmune or Po absorbed antisera, Schwann cells and myelin were not labeled. Since the sugar moiety of Po protein has been localized previously at the extracellular side or less dense line of PNS myelin, our results suggest that Po protein spans the membrane bilayer and may play a critical role in myelin compaction.

### 3. Immunocytochemical Localization of Myelin Associated Glycoprotein In Epon Sections of Developing Rat Peripheral Nerve

Myelin associated glycoprotein (MAG) is an integral membrane protein (MW  $\approx$  100,000) which is a minor component of purified PNS myelin. In previous immunocytochemical studies, MAG was localized in periaxonal, Schmidt-Lanterman incisures, and paranodal regions of PNS myelin. Compact regions of PNS myelin sheaths did not react with MAG antiserum. In the present study MAG was localized in 1  $\mu$ m thick Epon sections of developing and adult rat peripheral nerves and its localization was compared to that of the major structural protein (Po) of PNS myelin. To determine membrane specific localization of MAG, immunostained areas in one micron sections were traced on electron micrographs of adjacent thin sections. In addition to the periaxonal, Schmidt-Lanterman incisures and paranodal localization, MAG was present in the outer mesaxon of actively myelinating Schwann cells. These results demonstrate MAG's presence in "semicompact" Schwann cell membranes which have a gap of 5nm or more between cytoplasmic leaflets and a spacing of 12-14 nm between extracellular leaflets. In compact regions of the myelin sheath which do not contain MAG, the cytoplasmic leaflets of myelin membranes appear "fused" and form the major dense line while the extracellular leaflets are separated by a 1.5 nm gap appearing as paired minor dense lines. MAG may play a role in maintaining the periaxonal space, Schmidt-Lanterman incisures, paranodal myelin loops and outer mesaxons by preventing "complete" compaction of Schwann cell membranes. The presence of MAG in the outer mesaxon also suggests that MAG may serve a function in regulating myelination in the PNS.

In vitro: Rat SC in isolation only express myelin proteins temporarily, but maintain some other signs of differentiation such as phenotype, group organization and constant number of pulsations per day and have intense migratory activity. This motility behavior is preserved in secondary SC after mitogenic stimulation. This year we have studied the effects of fibronectin on rat SC and also explored their ability to interact and myelinate central axons.

### 1. Fibronectin Promotes Rat Schwann Cell Growth and Motility

Fibronectin (FN), a large cell surface protein which promotes fibroblast adhesion and migration, is also associated with perineurial cells in rat nerve. Cultures of dissociated newborn rat sciatic nerve were double-labeled with anti-FN serum and antibody to Thy 1.1 antigen, a rat fibroblast surface marker. Fibroblasts exhibit granular cytoplasmic FN, as well as fibrillar surface FN, whereas Schwann cells (SC) rarely express FN. When stable primary SC are treated with 20 to 40  $\mu$ g/ml of purified cell surface FN, they cluster progressively in small groups and their surfaces become covered with bound FN. SC growth was measured by  $^3$ H-thymidine incorporation during a 24h pulse and calculation of stimulation index (S.I. = % of labeled nuclei in stimulated cultures / % of labeled cells in unstimulated cultures). Mean SI of FN on primary SC was 3.0. Effect of identical FN treatment on purified unstimulated secondary SC was compared to that of known mitogenic factors. SI of bovine pituitary extract (BPE, 5  $\mu$ g/ml) is 2.2, of cholera toxin (CT, 1  $\mu$ g/ml) is

3.3, BPE and CT together is 5.3, of FN (20 µg/ml) is 4.3 and of FN and CT together is 4.5. FN thus has a clear effect on purified SC growth, as has been shown on neuroblastoma cells grown in serum-free medium (Exp. Cell Research 129, 361, 1980). In addition, FN triggers a 30 to 40-fold increase of directed migration of both primary and secondary SC in the Boyden chamber assay. Since it can promote migration and proliferation of SC *in vitro*, we suggest that FN, normally produced by perineurial cells *in vivo*, may play a role during nerve repair.

## 2. Study Cultured SC which are induced to Myelinate Axons

The interaction of subcultured SC with CNS axons has been investigated in mixed CNS aggregates. Preliminary studies of aggregates reveal Po staining of myelin sheaths three weeks after mixing the two cell populations and islands of SC which had acquired a basement membrane. The latter is never seen in isolated SC and its formation is known to be triggered by axonal contact. Interactions of cultured SC with peripheral axons is being investigated by grafting millions of rat SC into mouse transected sciatic nerve. These nerves have regenerated and we are trying to determine if our cultured rat SC, rather than the host SC, have remyelinated these axons.

3. Biological Compounds and Drugs can be tested on purified SC to see if they modify isolated SC structure, mitosis or motility. We have given frozen SC to investigators interested in these questions and have tested the effects of IDPN, a drug causing proximal axonal swellings and chronic demyelination in rat nerves. Alteration of the shape, motility and intermediate filament organization is reversibly produced in SC by this drug.

## Significance of the Program to the Institute:

Schwann cells can be easily stimulated to divide and myelinate. They have extensive repair capabilities both in the peripheral and central nervous system. Therefore, our experimental studies are relevant to the understanding of repair mechanisms in human demyelinating diseases such as multiple sclerosis and Guillain-Barre syndrome. In addition, our knowledge of which proteins are specific to CNS or PNS myelin is extending and the biological function of these specific proteins is becoming more clear through immuno-cytochemical studies. This will lead to better understanding of myelin compaction and organization which is so crucial to a normal conduction in myelinated axons.

## Proposed Course of the Project

Most of our future efforts will be directed towards the use of mixed CNS aggregates as a model system to study myelination of central neurons by SC. We will investigate sequentially the localization and emergence of proteins which are specific to either central or peripheral myelin. The mixed aggregates will be compared to CNS aggregates where only central myelin is formed. In addition, we are studying SC which develop and differentiate in dorsal root ganglia cultures. We are presently studying with phase and VIM the growth of

axons, the migration of SC along them and the relation between pulsations and ongoing myelination. VIM fluorescence will also be used if we can successfully raise an antibody to SC membrane and label this antibody with rhodamine. Subsequently, the major myelin proteins will be stained by immunolabeling of the fixed cultures as performed on nerve. Once the normal motility behavior and myelin protein emergence is sequentially analyzed in myelinating cultures, we will explore in this system (1) the glycoprotein function and transport, (2) the events related to remyelination after lysolecithin-induced demyelination, and (3) the effects of persistent viral infections on the structure and function of myelin-forming cells. We also plan to study the alteration of central myelin proteins expression in human viral diseases in which the oligodendrocytes is the first target such as PML (progressive multifocal leucoencephalopathy) caused by papova virus JC.

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Greenstein, J.I., Baron-Van Evercooren, A.G.S., Lazzarini, R.A. and McFarland, H.F.: Infection of the central nervous system produced by R<sub>1</sub> vesicular stomatitis virus (R<sub>1</sub>VSV). 1981. In Press.

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## ANNUAL REPORT

October 1, 1980 through September 30, 1981

Experimental Therapeutics Branch  
National Institute of Neurological and Communicative Disorders and Stroke

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ANNUAL REPORT  
October 1, 1980 through September 30, 1981  
Experimental Therapeutics Branch, IRP  
National Institute of Neurological and Communicative Disorders and Stroke  
Donald B. Calne, D.M., F.R.C.P., Chief

There has been no change in the organization of the Experimental Therapeutics Branch. It continues to conduct research primarily related to the treatment of neurological diseases. The resources available to the Branch have been sustained at an approximately steady level.

Therapeutics Section

1. Antiparkinson Efficacy of Lisuride

We have extended the pilot study on lisuride reported last year. We have treated 28 patients in a double blind cross-over comparison between bromocriptine and lisuride. There was no significant difference in the benefit derived by either drug, and adverse effects were also similar. The tendency for somnolence to be more troublesome in our initial studies was not confirmed when experience was extended. We now have 20 patients continuing on lisuride over a period up to 20 months, since they consider that this drug provides better control of their symptoms than had hitherto been attainable.

Pharmacokinetic studies have also been undertaken with lisuride. A tenfold difference in plasma concentration of the drug has been found in patients given the same dose. This indicates that there is substantial variation in the pharmacokinetic pattern for different individuals. The mean plasma half life of lisuride was 1.7 hours.

2. Antiparkinson Efficacy of Pergolide

A double blind, within-patient study of a new dopaminergic ergot derivative, pergolide, has been undertaken in Parkinsonian patients. Initial results indicate that this drug is a potent antiparkinson agent, which seems to be tolerated well.

3. Objective Tests of Parkinsonian Deficits

We have compared the results of subjective clinical scoring of neurological deficits with objective measurements of reaction time, speed of movement of the hand, mean length of step when walking, and mean duration of steps. Subjective clinical scoring techniques proved more sensitive than objective measurements, but the latter offered more consistent results, which were easier to store, analyze and retrieve. This work was undertaken in collaboration with Dr. E. Evarts of NIMH.

4. Decarboxylation of Levodopa in Man

The technique reported last year (collection of  $C^{14}O_2$  after injection of  $C^{14}$  carboxyl labelled levodopa) has been applied to the problem of why certain patients undergo severe fluctuations in response (on-off phenomenon and wearing-off reactions). It has been found that fluctuations in response to levodopa are not related to changes in the rate of decarboxylation. This work resulted from collaboration with Dr. I. Kopin of NIMH.

## 5. Twin Studies

Our study of Parkinson's disease in twins has been extended to include the examination of 47 twin pairs. There were 33 monozygotic and 14 dizygotic pairs. Definite concordance was only seen in one pair of monozygotic twins; there was no case of concordance in the dizygotic pair. This work has been performed in collaboration with Dr. R. Eldridge, NINCDS, and Dr. R. Duvoisin, Rutgers Medical School.

## 6. Adverse Reactions to Bromocriptine

Following a report of pulmonary complications of bromocriptine therapy, 50 patients who have taken this drug for periods up to 6 years have been reviewed. Chest x-rays have not undergone any change during treatment with bromocriptine in 49 cases, but one patient developed a pleural effusion with pulmonary and pleural fibrosis. These changes improved markedly when treatment with bromocriptine was stopped, and pulmonary function has returned to normal.

## 7. Studies with Deprenyl

Our previous studies on deprenyl in Parkinson's disease indicated that this drug might have some euphoriant effect, but antiparkinson efficacy was not detected. From a biochemical survey of our patients during deprenyl therapy, it appeared that amphetamine formed by metabolic transformation of deprenyl may have contributed to an alteration in mood. This work was performed in collaboration with Dr. F. Karoum of NIMH.

# Pharmacology Section

## 1. Positron Emission Tomographic Studies

Huntington's chorea and Alzheimer's disease patients are currently being studied by positron emission tomography following  $^{18}\text{F}$ -2-fluoro-2-deoxyglucose administration to correlate aspects of cognitive function with regional cortical activity. Due to a shortage of labeled deoxyglucose, only one patient could be scanned during the past year. Future plans call for an acceleration of this work together with implementation of a recently approved protocol to examine hyperkinetic extrapyramidal disorders where no pathologic lesions have been found to account for neurologic symptoms.

## 2. Cerebrospinal Fluid Studies

These studies have recently focused on the use of oxygen-18 labeling to assess central monoaminergic function. Previous investigations suggested that parkinsonian patients have a relatively small but rapidly turning-over pool of dopamine in comparison with individuals with Huntington's chorea. Efforts during the past year have been directed towards the acquisition of normative data; the results are consistent with our earlier findings.

## 3. Dopamine Agonist Therapy

These investigations address the hypothesis that dopamine agonists which preferentially stimulate dopamine autoreceptors may inhibit dopaminergic transmission and thus

diminish symptoms reflecting hyperfunction of this system. Since data supporting this contention in part derive from our experience with apomorphine, recent studies have primarily involved n-propylnorapomorphine, a relatively non-toxic apomorphine derivative, suitable for oral administration. Initial results suggest this drug may be safe and effective in patients with tardive dyskinesia and schizophrenia.

#### 4. GABA-mimetic Therapy

Based on clinical and preclinical observations indicating that augmentation of GABA-mediated synaptic function may benefit patients with tardive dyskinesia and related hyperkinetic extrapyramidal disorders, clinical studies have been initiated with several novel GABA-mimetic compounds including  $\lambda$ -vinyl GABA, which elevates brain GABA by inhibiting its degradatory enzyme, and 4,5,6,7-tetrahydroisoxazolo-(5,4-c) pyridine-3-ol (THIP), which acts as a GABA agonist. Preliminary results are encouraging.

#### 5. Endorphin Studies

$\beta$ -lipotropin fragments such as destyrosine- $\lambda$ -endorphin ( $\beta$ -LPH<sub>62-77</sub>; DT $\lambda$ E) appear to exhibit neuroleptic-like activity in the experimental animal, without directly affecting dopaminergic transmission. We have recently completed an evaluation of DT $\lambda$ E in chronic schizophrenic subjects, some of whom also had tardive dyskinesia. Results of this controlled trial failed to reveal any consistent alteration in behavioral, motor, or endocrinologic function.

#### 6. Vasopressin Studies

Preclinical observations implicate vasopressin in the regulation of mammalian learning and memory. During the past year we completed a controlled study of lysine vasopressin in Alzheimer's disease patients. There was no measurable improvement in cognitive function, although one test of reaction time yielded results consistent with an overall altering action.

#### 7. Neuroendocrine Studies

Recent endocrinologic probes have attempted to elucidate the extent of hypothalamic neuronal dysfunction in Huntington's chorea. We have found that Huntingtonian women have an elevated daily secretion of growth hormone, an exaggerated growth hormone response to dopamine agonists, and a blunted response to a dopamine antagonist. Although these results suggest hypothalamic-pituitary dopamine system hyperfunction, preliminary data now indicate growth hormone hyperresponsivity also occurs with drugs which selectively affect nondopaminergic systems.

#### 8. Substance P Studies

Interest in this peptide derives from the strategic localization of substance P systems in relation to dopaminergic projections between substantia nigra and corpus striatum. Preliminary results in mice suggest that systemically administered substance P increases latency (improves memory) in passive avoidance learning paradigms, inhibits electrocortical shock-induced amnesia, potentiates amphetamine-induced stereotypy, and possibly exerts an analgesic effect.

## 9. Cholecystokinin Studies

Cholecystokinin appears to act as a neuromediator in some dopamine-containing neurons; systemic administration of cholecystokinin might thus influence central dopaminergic function. Consistent with the view that this peptide may possess neuroleptic-like (dopamine receptor blocking) activity, we found that cholecystokinin octapeptide (CCK-8) diminished apomorphine-induced rodent motor activity and reduced signaled-avoidance behavior in a dose-dependent manner. Moreover, CCK-8 potentiated the ability of a neuroleptic, haloperidol, to impair avoidance responses.

## Clinical Epilepsy Section

### I. Diagnostic and Therapeutic Reevaluation of Patients with Intractable Epilepsy

The Clinical Epilepsy Section is using recently developed techniques of intensive monitoring to achieve improved seizure control, reduction of drug-induced side effects, and better potential for rehabilitation. These include simultaneous video and telemetered EEG recording of seizures, and daily determinations of antiepileptic drug serum concentrations.

Specific studies involve seizure classification and differentiation of frequently confused seizure types. A detailed study of the clinical characteristics of 163 complex partial seizures was performed. These features can be used to distinguish complex partial seizures from other seizure types on clinical grounds alone. A small number of patients with psychogenic seizures have also been studied. These are very difficult to distinguish from true epilepsy during outpatient evaluation. A study of 36 videotaped generalized tonic-clonic seizures showed that most were the result of secondary generalization of partial seizures.

The long term benefit of intensive monitoring has been shown in a follow-up study of 74 patients. Sixty-nine percent maintained improvement in at least one therapeutic modality an average of 25 months after discharge. Thirty-eight percent showed significant social and behavioral improvement. New diagnostic techniques are also being evaluated. Positron emission tomography using  $F^{18}$  2-deoxyglucose is an important development. Initial studies have suggested that patients with partial seizures have focal hypometabolic cerebral areas, corresponding to the interictal seizure EEG focus. During a seizure, this hypometabolic region is converted into a hypermetabolic one. In addition, an occasional patient with diffuse EEG abnormalities may have a highly focal scan using the positron emission tomography technique. PET scanning may obviate the need for depth electrode study in patients with medically intractable seizures being considered for surgical therapy.

The recent utilization of evoked responses in patients with epilepsy has opened a new field for studies of patients with intractable seizures. Preliminary data suggest that the dominant eye may greatly influence the amplitude of the visual evoked response. These subtle changes may be wrongly interpreted in patients with partial seizures unless this asymmetry is accounted for. In addition, patients with complex partial seizures are currently being evaluated for abnormalities of the visual evoked response, auditory and brainstem evoked potentials, and the somatosensory evoked potentials.



The video-taped seizures at the Clinical Epilepsy Section have formed the basis of an unparalleled library of seizures for teaching and analysis. They formed the integral part of educational films such as, "Differential Diagnosis of Complex Partial Seizures", and "Classification of Epileptic Seizures". The Clinical Epilepsy Section is constantly making technical advances in intensive monitoring.

## 2. Clinical Pharmacology of Antiepileptic Drugs

Pharmacologic projects are underway and are described in the following paragraphs.

Progabide, a new drug being evaluated for epilepsy has been tested in eight European countries. Studies are ongoing with normal volunteers to evaluate a new micronized formulation and will be followed by pilot studies in patients with epilepsy. The drug is a putative GABA agonist and its mechanism of action may be through its effect on this inhibitory transmitter.

The effect of taking medication with and without food has been studied in patients in the Clinical Epilepsy Section. The specific drug under study thus far, carbamazepine, has demonstrated little change in steady-state levels, but some changes are demonstrable in the rate of absorption in a few patients.

A study has been completed on the evaluation of the removal of sedative hypnotic antiepileptic drugs. Barbiturates and benzodiazepines were completely withdrawn from patients with intractable seizures. In spite of the removal of these antiepileptic drugs which increase medication toxicity, patients were largely improved with regard to their seizure control and most patients reported some improvement in toxic side effects.

A number of studies have been done in drug-drug interactions of antiepileptic drugs. A study of the interaction between phenytoin and primidone demonstrated that metabolite levels of primidone are altered by the phenytoin, with the major effect being a direct inhibition of the metabolite phenobarbital by phenytoin. Another metabolite of primidone, phenylethylmelanamide was not altered. In a different study, the interaction between valproic acid and phenobarbital was studied in which valproic acid also inhibited phenobarbital metabolism. In order to evaluate the mechanism of this effect, the influence of valproic acid on acetaminophen was studied, and this demonstrated that the effect on phenobarbital is likely to be inhibition of hydroxylation rather than glucuronidation. A study is planned to evaluate the effect of carbamazepine on phenytoin; this will be carried out using heavy-labeled phenytoin in which the pharmacokinetic parameters of phenytoin will be determined before and after the addition of carbamazepine. These results will be useful in understanding the mechanisms by which carbamazepine interacts and changes the plasma levels of phenytoin when these drugs are given in combination.

## Biochemical Neuropharmacology Section

### 1. Biochemical Studies of Dopaminergic Receptors

In the past year the Biochemical Neuropharmacology Section has continued to investigate the dopamine receptor in the intermediate lobe (IL) of the rat pituitary gland. The knowledge obtained from these investigations has been applied to investigations of the dopamine receptors in the brain.



The IL contains a D-2 receptor amenable to experimental investigation. By histological or cytochemical criteria, the intermediate lobe is a relatively homogeneous tissue synthesizing and secreting alpha-melanocyte stimulating hormone (alpha-MSH) and other hormones related to proopiomelanocortin. The physiological response of the intermediate lobe to dopamine, an inhibition of the release of alpha-MSH, can be easily quantified. A beta-adrenoceptor occurs on the parenchymal cells of the intermediate lobe. The available evidence suggests that occupancy by agonists (i.e. stimulation) of the beta-adrenoceptor enhances adenylate cyclase activity, resulting in an accumulation of cyclic AMP which initiates the intracellular events ultimately expressed as the enhanced release of alpha-MSH.

In the past fiscal year, the involvement of calcium ions in regulating the synthesis of cAMP and release of alpha-MSH were studied. Calcium was not obligatory for the enhancement of cAMP synthesis by beta-adrenergic agonists, phosphodiesterase inhibitors or cholera toxin; however, the ion was obligatory for the stimulation of release of alpha-MSH by any of these agents or by 8Bromo-cAMP. Under certain circumstances, calcium ions inhibit synthesis of cAMP. The beta-adrenoceptor appears to be coupled to adenylate cyclase by a stimulatory guanyl nucleotide regulatory constituent (designated as  $N_s$ ). Stimulation of the beta-adrenoceptor alters  $N_s$  so that GTP can interact with  $N_s$  and enhance adenylate cyclase activity.

The dopamine receptor in the intermediate lobe has been extensively studied in the past fiscal year. Dopamine diminishes the basal release of alpha-MSH as well as the L-isoproterenol-induced accumulation of cyclic AMP and the L-isoproterenol-enhanced release of alpha-MSH. Following treatment of IL tissue with cholera toxin (a non-specific activator of adenylate cyclase) IL tissue has higher adenylate-cyclase activity and releases more alpha-MSH; dopamine markedly decreases adenylate-cyclase activity, cAMP accumulation and alpha-MSH release from CT treated tissue. The pharmacology of the dopamine-receptor can be characterized on the basis of the ability of drugs to mimic or block these effects of dopamine upon cholera toxin treated tissue; however, the dopaminergic inhibition of adenylate cyclase in cholera toxin-treated IL tissue and the dopamine-induced decrease in the responsiveness of the beta-adrenoceptor have provided the basis for our characterization of the pharmacology of the dopamine-receptor. The dopamine-receptor recognizes dopamine, apomorphine and the dopaminergic ergots as agonists, and is antagonized by a variety of dopamine antagonists, including (-) sulpiride, a substituted benzamide which selectively blocks D-2 receptors; therefore, the dopamine-receptor in the IL has been assigned to the D-2 category. The biochemical basis of the decreased responsiveness of the beta-adrenoceptor was investigated; apomorphine was the dopaminergic agonist of choice for these studies because it does not interact with the beta-adrenoceptor. The D-2 receptor appears to be coupled to adenylate cyclase by an inhibitory guanyl nucleotide regulatory constituent designated as  $N_i$ . Occupancy of the receptor by agonists (i.e. stimulation) alters the properties of  $N_i$  so that GTP can interact with  $N_i$  and cause an inhibition of adenylate cyclase. These investigations were culminated by the identification of compound LY-141865 as a specific, selective agonist upon the D-2 receptor (devoid of agonist or antagonist actions on the D-1 receptor).

Based on the results obtained in intermediate lobe, the possibility that a D-2 receptor inhibiting cAMP formation exists in the striatum of the rat brain. The presence of a D-1 receptor stimulating cAMP formation had been previously demonstrated in several laboratories. Using LY-141865, a selective D-2 agonist, and SK&F 38393, a selective D-

l agonist, dopaminergic inhibition of cAMP synthesis was demonstrated. Dopamine itself stimulates both categories of receptor.

## Physiological Neuropharmacology Section

### I. Physiological Effects of Dopamine and Dopamine Agonists in the Basal Ganglia

To gain insight into how dopamine and the dopamine agonists affect information processing in the basal ganglia, we have been examining the effects of these agents at sites where information funnels out of the basal ganglia; the pars reticulata of the substantia nigra and the globus pallidus.

In the pars reticulata of the substantia nigra, cells are heavily innervated by striatal efferents, and are thus in a position to be affected indirectly by the action of dopamine and dopamine agonists in the striatum. In addition, it has been postulated that dopamine neurons may release dopamine from their dendrites in the pars reticulata and, therefore, may also directly affect the activity of the reticulata neurons. We have begun to investigate these possible influences of dopamine on rat reticulata cell activity by studying the responses of the reticulata cells to iontophoresed dopamine. In addition, the ability of dopamine to alter the effects of iontophoresed GABA and glycine has also been examined. We have found that dopamine alone causes increases in reticulata cell activity. Moreover, dopamine consistently and markedly attenuates the effects of iontophoresed GABA but not glycine on these neurons. Norepinephrine does not consistently alter the absolute amount of inhibition elicited by either GABA or glycine. Sixty percent of the cells studied in these experiments could be antidromically activated from the ipsilateral ventromedial nucleus of the thalamus. Thus, these results indicate that dopamine, released from the dendrites within the nigra, and systemically administered dopamine agonists, could potentially modulate the actions of GABA on substantia nigra pars reticulata neurons projecting to the ventromedial nucleus of the thalamus and to other areas. The increased firing elicited by dopamine alone may reflect either the neurotransmitter's direct excitatory action and/or its ability to modulate inhibitory influences provided by the GABAergic inputs these cells receive. This demonstration of dopamine's ability to modulate GABA's inhibitory effects on neuronal activity provides the basis for hypothesizing a novel and potentially very significant mechanism through which dopamine and dopamine agonists may act to alter information processing in the basal ganglia.

We have also been interested in determining how dopaminergic drugs affect globus pallidus (external pallidus) activity in the rat. Anatomical considerations suggest that the actions of dopamine on dopamine autoreceptors, on dopamine receptors in the striatum and, possibly, on dopamine receptors in the globus pallidus, should ultimately summate to affect the activity of cells in the globus pallidus. We found that systemic administration of d-amphetamine causes significant increases in the unit activity of spontaneously firing neurons in this area. This action seems related to the ability of d-amphetamine to release dopamine since l-amphetamine, which produces similar effects on norepinephrine release but smaller effects on dopamine release caused only a slight excitation of pallidal neurons. The serotonin uptake inhibitor fluoxetine produced only varied changes in firing, while minor effects were also observed after systemic administration of desmethylimipramine and clonidine. These results suggest that amphetamine-induced release of dopamine is associated with a marked stimulation of firing rates of spontaneously active globus pallidus cells in qallamine-paralyzed rats.

It has been hypothesized that some of the paradoxical or biphasic behavioral effects of dopamine agonists, such as apomorphine, reflect differences in the relative sensitivity of different dopamine receptors. Low doses of apomorphine appear to act preferentially at the dopamine autoreceptor to decrease nigral dopamine cell activity and dopamine release, causing a net decrease in postsynaptic dopamine receptor stimulation, whereas larger doses of apomorphine stimulate postsynaptic dopamine receptors. Since it seemed possible that the tonic activity of globus pallidus neurons would be influenced by the level of postsynaptic dopamine receptor stimulation and might reflect these hypothesized biphasic effects of dopamine agonists interacting with the various sub-categories of pre- and post-synaptic dopamine receptors in the basal ganglia and substantia nigra, we examined the effects of systemic administration of a wide range of doses of apomorphine on pallidal activity. Like amphetamine, this agonist, in doses of .08 to 1.0 mg/kg, markedly enhanced the firing of spontaneously active pallidal neurons. However, low doses (5, 20  $\mu$ g/kg) which preferentially stimulate presynaptic dopamine receptors did not cause effects opposite to those observed with larger doses in 96% of the cells monitored. Thus, the paradoxical effects of low doses of dopamine agonists are not reflected in alterations in the tonic activity of cells in the globus pallidus.

On the other hand, additional observations made during these studies may provide new clues about some of the paradoxical effects of these drugs. Our findings show that the expression and magnitude of the excitation induced by apomorphine are influenced by the schedule of drug administration. When a nonexcitatory, small dose of apomorphine is given before a dose which, by itself, caused a maximal stimulation of pallidal activity, the first dose appears to have a priming effect, altering or setting the systems response, so that additional injections of apomorphine have little further effect. Thus, the method of apomorphine administration should be taken into consideration when the effects of this drug on brain metabolism, animal behavior, and possibly, therapeutic potential are being investigated. The results also raise the possibility that some of the useful paradoxical effects of dopamine agonists in hyperkinetic disorders may be related to the "priming effects" of low doses that were observed in these studies.

Other dopamine agonists which were found to cause consistent increases in the activity of pallidal neurons are lisuride and pergolide. While many of these effects of the dopamine agonists on pallidal activity may be indirect, mediated through interactions of the drugs with dopamine receptors in the striatum on neurons projecting to the external pallidus, we observed that dopamine iontophoresed onto cells in the globus pallidus, as in the substantia nigra pars reticulata, partially blocked the ability of GABA to inhibit pallidal activity. Since there is large GABAergic innervation of the globus pallidus cells, originating in the striatum, this observation suggests that the dopamine agonists may also increase pallidal activity by attenuating the effects of GABA on these neurons. Neither norepinephrine nor acetylcholine had similar effects on GABA's ability to inhibit pallidal activity indicating that this effect is, at least to some extent, specific for dopamine.

The Section has also been conducting an investigation of the effects of subchronic administration of L-dopa (2 injections/day for 5-12 days) on the ability of dopamine neurons to respond to apomorphine, amphetamine and L-dopa. These studies should provide insight into the effects of this treatment on the sensitivity of dopamine autoreceptors and the dynamics of dopamine synthesis and turnover. To date, the results do not support the observations which have appeared from time to time in the literature suggesting that dopamine agonists cause dopamine receptor supersensitivity. We have found either a decrease or no change in the responses of dopamine neurons to dopaminomimetics after subchronic L-dopa treatment.



## 2. Physiological Effects of GABA and GABA Agonists in the Basal Ganglia

Previously we have shown that the cells in the substantia nigra pars reticulata are sensitive to the inhibitory effects of both i.v. muscimol, a potent GABA agonist, and iontophoretically applied GABA and muscimol. Since it has been postulated that the central effects of the benzodiazepines are mediated by the ability of the benzodiazepines to potentiate the inhibitory effects of GABA on neuronal activity, we have examined the actions of diazepam and flurazepam on activity of reticulate cells. When administered systemically, both drugs exerted inhibitory effects on the activity of these neurons which are of the same order of magnitude as those which have been reported for locus coeruleus neurons. These studies suggest that the benzodiazepines may have multiple clinically relevant sites of action.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01-NS 02258-05 ET
PERIOD COVERED <p style="text-align: center;">October 1, 1980 through September 30, 1981</p>		
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Therapeutic Studies in Parkinsonism and Other Movement Disorders</p>		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	D.B. Calne C. Ward P. Le Witt A. Larsen	Chief, Therapeutics Section Clinical Associate Clinical Associate Visiting Fellow
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Other:	I. Kopin E. Evarts F. Karoum W. Lovenberg  R. Eldridge	Senior Psychiatrist Chief Chemist Chief, Biochemical Pharmacology Section Head, Clinical Neurogenetic Studies
		LCS LNP DSMR HE  NES
		NIMH NIMH NIMH NHLBI  NINCDS
COOPERATING UNITS (if any)    Laboratory of Clinical Science, NIMH; Adult Psychiatry Branch, Division of Special Mental Health Research, NIMH; Laboratory of Neurophysiology, NIMH; Biochemical Pharmacology Section, HE, NHLBI; Department of Neurology, Rutgers University School of Medicine.		
LAB/BRANCH Experimental Therapeutics Branch		
SECTION Therapeutics Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: <p style="text-align: center;">4.5</p>	PROFESSIONAL: <p style="text-align: center;">4.5</p>	OTHER: <p style="text-align: center;">0</p>
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The purpose of this study is to investigate the possible <u>efficacy</u> and <u>safety</u> of new compounds applied to the treatment of certain disorders of movement, and to employ drugs as tools to analyze the physiological and pharmacological mechanisms mediating various motor deficits.  The main conclusions deriving from observations over the last year are: (1) the antiparkinson efficacy of <u>lisuride</u> has been confirmed, and the response was not associated with excessive somnolence; (2) a new dopaminergic ergot derivative, <u>pergolide</u> , has been found to possess antiparkinson properties; (3) a comparison between <u>subjective</u> and <u>objective</u> methods of measuring neurological deficits in movement disorders indicated that subjective tests are more sensitive, while objective tests are more consistent, and are easier to store, analyse and retrieve; (4) fluctuations in the clinical response to levodopa have been studied by monitoring <u>decarboxylation</u> ; there is <u>no correlation</u> between changes in Parkinsonian signs and alteration in the rate of <u>decarboxylation</u> ; (5) in a retrospective study of adverse reactions to <u>bromocriptine</u> , 1 out of 50 patients was found to have developed pleural effusions and <u>pleuropulmonary fibrosis</u> ; this was reversible; (6) metabolic studies indicate that the euphoriant effect of <u>deprenyl</u> may derive, in part, from the formation of <u>amphetamine</u> .		



Project Description:Objectives:

This project is designed to improve treatment and elucidate the pharmacological, physiological and biochemical abnormalities occurring at synaptic level in certain neurological diseases.

Methods:

Inpatients and outpatients are studied. Specimens of body fluids (including CSF) are taken for assay of transmitters, their metabolites, drugs, and routine biochemical and haematological indices of pharmacotoxicity. Motor control is studied by measuring velocity and force of movement, integrated electromyographic activity, and by conventional clinical scoring techniques that involve careful history taking and physical examination. Where possible the patient is used as his own control by making observations during different (blind) therapeutic regimens, and by studying asymmetric motor deficits.

Major Findings and Significance to Biomedical Research and the Program of the Institute:1) Antiparkinson Efficacy of Lisuride

We have extended the pilot study on lisuride reported last year. We have treated 28 patients in a double blind cross-over comparison between bromocriptine and lisuride. There was no significant difference in the benefit derived by either drug, and adverse effects were also similar. The tendency for somnolence to be more troublesome in our initial studies was not confirmed when experience was extended. We now have 20 patients continuing on lisuride over a period up to 20 months, since they consider that this drug provides better control of their symptoms than had hitherto been attainable.

Pharmacokinetic studies have also been undertaken with lisuride. A tenfold difference in plasma concentration of the drug has been found in patients given the same dose. This indicates that there is substantial variation in the pharmacokinetic pattern for different individuals. The mean plasma half life of lisuride was 1.7 hours.

2) Antiparkinson Efficacy of Pergolide

A double blind, within patient study of a new dopaminergic ergot derivative, pergolide, has been undertaken in Parkinsonian patients. Initial results indicate that this drug is a potent antiparkinson agent, which seems to be tolerated well.

3) Objective Tests of Parkinsonian Deficits

We have compared the results of subjective clinical scoring of neurological deficits with objective measurements of reaction time, speed of movement of the hand, mean length of step when walking, and mean duration of steps. Subjective clinical scoring techniques proved more sensitive than objective measurements, but the latter offered more consistent results, which were easier to store, analyze and retrieve. This work was undertaken in collaboration with Dr. E. Evarts of NIMH.

4) Decarboxylation of Levodopa in Man

The technique reported last year (collection of  $C^{14}O_2$  after injection of  $C^{14}$  carboxyl-labelled levodopa) has been applied to the problem of why certain patients undergo severe fluctuations in response (on-off phenomenon and wearing off reactions). It has been found that fluctuations in response to levodopa are not related to changes in the rate of decarboxylation. This work resulted from collaboration with Dr. I. Kopin of NIMH.

5) Twin Studies

Our study of Parkinson's disease in twins has been extended to include the examination of 47 twin pairs. There were 33 monozygotic and 14 dizygotic pairs. Definite concordance was only seen in one pair of monozygotic twins; there was no case of concordance in the dizygotic pair. This work has been performed in collaboration with Dr. R. Eldridge, NINCDS, and Dr. R. Duvoisin, Rutgers Medical School.

6) Adverse Reactions to Bromocriptine

Following a report of pulmonary complications of bromocriptine therapy, 50 patients who have taken this drug for periods up to 6 years have been reviewed. Chest x-rays have not undergone any change during treatment with bromocriptine in 49 cases, but one patient developed a pleural effusion with pulmonary and pleural fibrosis. These changes improved markedly when treatment with bromocriptine was stopped, and pulmonary function has returned to normal.

7) Studies with Deprenyl

Our previous studies on deprenyl in Parkinson's disease indicated that this drug might have some euphoriant effect, but antiparkinson efficacy was not detected. From a biochemical survey of our patients during deprenyl therapy, it appeared that amphetamine formed by metabolic transformation of deprenyl may have contributed to an alteration in mood. This work was performed in collaboration with Dr. F. Karoum of NIMH.

Proposed Course:

1) The efficacy and toxicity of pergolide will be evaluated in a double blind controlled study.

2) A new D-1 agonist, SK and F 38393, will be investigated as a potential antiparkinson agent.

3) Epidemiological studies will be undertaken to evaluate risk factors in the etiology of Parkinson's disease.

4) PET scanning will be applied to the problem of identifying regional changes in function of the basal ganglia in patients with disorders of movement.

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CT No. 80-N-41  
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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02265-05 ET																
PERIOD COVERED <p style="text-align: center;">October 1, 1980 to September 30, 1981</p>																		
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Pharmacology, Biochemistry and Physiology of Central Neurotransmitters</p>																		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 15%;">PI:</td> <td style="width: 35%;">Thomas N. Chase</td> <td style="width: 40%;">Chief, Pharmacology Section</td> <td style="width: 10%;">ETB NINCDS</td> </tr> <tr> <td>OTHER:</td> <td>Raymon Durso</td> <td>Clinical Associate</td> <td>ETB NINCDS</td> </tr> <tr> <td></td> <td>Marth Knight</td> <td>Staff Fellow</td> <td>ETB NINCDS</td> </tr> <tr> <td></td> <td>Carol Tamminga</td> <td>Guest Worker</td> <td>ETB NINCDS</td> </tr> </table>			PI:	Thomas N. Chase	Chief, Pharmacology Section	ETB NINCDS	OTHER:	Raymon Durso	Clinical Associate	ETB NINCDS		Marth Knight	Staff Fellow	ETB NINCDS		Carol Tamminga	Guest Worker	ETB NINCDS
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COOPERATING UNITS (if any) <p style="text-align: center;">K. Schlesinger, University of Colorado; G. Sedvall, Karolinska Institute, Stockholm; D. Samuel, Weizmann Institute, Rehovot.</p>																		
LAB/BRANCH <p style="text-align: center;">Experimental Therapeutics Branch</p>																		
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INSTITUTE AND LOCATION <p style="text-align: center;">NINCDS, NIH, Bethesda, Maryland 20205</p>																		
TOTAL MANYEARS: <p style="text-align: center;">2.5</p>	PROFESSIONAL: <p style="text-align: center;">2.2</p>	OTHER: <p style="text-align: center;">.3</p>																
CHECK APPROPRIATE BOX(ES) <table style="width: 100%; border: none;"> <tr> <td><input checked="" type="checkbox"/> (a) HUMAN SUBJECTS</td> <td><input checked="" type="checkbox"/> (b) HUMAN TISSUES</td> <td><input type="checkbox"/> (c) NEITHER</td> </tr> <tr> <td colspan="3"><input type="checkbox"/> (a1) MINORS    <input checked="" type="checkbox"/> (a2) INTERVIEWS</td> </tr> </table>			<input checked="" type="checkbox"/> (a) HUMAN SUBJECTS	<input checked="" type="checkbox"/> (b) HUMAN TISSUES	<input type="checkbox"/> (c) NEITHER	<input type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS												
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<input type="checkbox"/> (a1) MINORS <input checked="" type="checkbox"/> (a2) INTERVIEWS																		
SUMMARY OF WORK (200 words or less - underline keywords)  <p>The goal of this project is to develop improved <u>drug therapies</u> for nervous system disease. Clinical and preclinical <u>investigations</u> seek to elucidate how the activity of specific <u>transmitter systems</u> relate to <u>neuropsychiatric</u> function. Based on these <u>relationships</u>, novel <u>pharmaceutical agents</u> are evaluated for their ability to influence central synaptic processes and thus modify neurologic symptoms. Major topics now under study include:</p> <ol style="list-style-type: none"> <li>1) Application of the <u>oxygen-18</u> technique to evaluate central monoaminergic mechanisms and the <u>Fluorodeoxyglucose</u> method combined with <u>positron emission tomography</u> to assess regional neuronal function in man.</li> <li>2) Relation of the <u>dopamine system</u> and of closely interactive transmitter systems to <u>extrapyramidal motor</u> function.</li> <li>3) Ability of selected agonists and antagonists of dopamine, <u>gamma aminobutyric acid</u>, and certain <u>peptides</u> to influence motor and <u>cognitive behavior</u>.</li> </ol>																		

Objectives and Methods Employed:

The Section directs its principal research efforts towards the rational development of improved drug treatments for nervous system disease. On the basis of an integrated program of clinical and preclinical studies, attempts are made to relate the activity of a particular transmitter system to a specific centrally mediated function. Novel pharmacologic agents are then evaluated in animal models and in man for their ability to selectively modify these systems and thus ameliorate neurologic or psychiatric symptoms. The major focus is on dopaminergic mechanisms in relation to hyperkinetic extrapyramidal disorders; the role of the dopamine system, as well as other transmitter systems with which it interacts, in the regulation of cognitive and endocrinologic function are also investigated.

Major Findings and Proposed Course:1. Positron Emission Tomographic Studies

Investigations of regional neuronal activity in patients with Huntington's chorea and Alzheimer's disease are currently being conducted by means of positron emission tomography (PET) following  $^{18}\text{-F-2-fluoro-2-deoxyglucose}$  administration. In part, these studies attempt to correlate various aspects of cognitive function - under basal conditions as well as during activation by mental tasks and drug treatment - with regional cortical activity. Due to a continuing shortage of the labeled glucose derivative, only one patient could be studied during the past year. This individual with Alzheimer's disease evidenced a marked constructural apraxia; and his PET scan showed a large area of neuronal hypofunction in the nondominant parietal lobe. Further tests of the participation of this region in various forms of apraxia are planned. In addition, a protocol has recently been approved to extend these studies to patients with certain hyperkinetic extrapyramidal disorders (tardive dyskinesia, Tourette syndrome, and dystonia musculorum deformans) where no pathologic lesions have been found to account for neurologic symptoms. Positive findings may help direct future biochemical probes of the pathogenesis of these disorders.

2. Cerebrospinal Fluid Studies

These clinical studies, conducted in collaboration with the Weitzman Institute and the Karolinska Institute, have recently focused on the use of oxygen- $^{18}$  labeling to estimate the functional state of central monoaminergic systems. Inhalation of a breathing mixture highly enriched in this stable (nonradioactive) isotope of oxygen produces readily detectable labeling of monoamine metabolites in lumbar cerebrospinal fluid. Previous investigations suggested that parkinsonian patients have a relatively small but rapidly turning over pool of central dopamine in comparison with individuals with Huntington's chorea. The possibility that a compensatory hyperactivity of residual dopamine neurons occurs in Parkinson's disease may have important implications for the development of improved drug therapies for this disorder. In order to better evaluate these results, efforts during the past year have been

directed towards the acquisition of normative data, mainly from asymptomatic individuals who are at risk for Huntington's disease. Due to inadequate supplies of oxygen-18, however, only a small number of individuals could be studied. Future plans call for the completion of this work together with an examination of the effects of dopamine agonists and antagonists (and possibly certain peptides) on central dopaminergic function.

### 3. Dopamine Agonist Therapy

Clinical studies during the past year have continued to evaluate the ability of dopamine receptor agonists to ameliorate hyperkinetic extrapyramidal disorders. These investigations are based on the hypothesis that dopamine agonists which preferentially stimulate dopamine autoreceptors might inhibit dopaminergic transmission and thus diminish neurologic and psychiatric symptoms reflecting hyperfunction of this system. Since data supporting this contention in part derive from previous experience with apomorphine, recent studies have primarily involved n-propylnorapomorphine, a relatively non-toxic apomorphine derivative, suitable for oral administration. Results collected thus far in a placebo controlled, double blind trial in eleven non-neuroleptic treated patients with tardive dyskinesia and schizophrenia appear most promising with respect to relief of both dyskinetic and psychotic symptoms. Preliminary controlled studies with another non-ergot dopamine agonist, S-3608, have also yielded promising results in seven tardive dyskinesia patients. Clinical experiments with both these drugs, conducted together with appropriate monitoring of their central biochemical actions, will be continued.

### 4. GABAmimetic therapy

Numerous biochemical and pharmacologic observations indicate that GABA system alterations may be associated with various central nervous system disorders, and suggest that augmentation of GABA-mediated synaptic function may benefit patients with tardive dyskinesia and related, naturally occurring or drug-induced hyperkinetic extrapyramidal disorders. In order to further evaluate the pathogenetic and therapeutic implications of the reduction in involuntary movements occurring in tardive dyskinesia patients during the administration of the potent GABA agonist, muscimol, clinical studies have recently been initiated with several novel GABAmimetic compounds: SL76002, a putative GABA agonist, has been approved for NIH study, but drug supplies sufficient for these investigations have not yet been made available;  $\lambda$ -vinyl GABA, which elevates brain GABA by inhibiting its degradatory enzyme, has just begun to be tested in tardive dyskinesia patients to ascertain safe and effective dose levels; THIP, a bicyclic congener of muscimol, has just been approved for NIH study and will soon receive investigative attention. Certain of these drugs will also be in combination with a short acting benzodiazepine derivative in an attempt to improve their therapeutic to toxic ratio.

## 5. Endorphin Studies

The limited antidyskinetic efficacy and potential central toxicity of neuroleptics, which presumably act through blockade of dopamine receptors, have prompted the search for alternative pharmacologic approaches to the treatment of hyperkinetic extrapyramidal disorders. Recent evidence suggests that fragments of the pituitary hormone  $\beta$ -lipotropin ( $\beta$ -LPH) exert behavioral effects not mediated by opiate receptors. One such compound, destyrosine- $\lambda$ -endorphin ( $\beta$ -LPH<sub>62-77</sub>; DT $\lambda$ E), exhibits neuroleptic-like activity in the experimental animal, but apparently without directly affecting dopaminergic transmission. Preliminary and largely uncontrolled clinical trials elsewhere have suggested that DT $\lambda$ E may possess antipsychotic activity. To evaluate both antidyskinetic and antipsychotic potency, we have administered DT $\lambda$ E subacutely to five male chronic schizophrenic subjects, some of whom also had tardive dyskinesia. Results of this double-blind, placebo-controlled trial failed to reveal any consistent alteration in behavioral, motor, or endocrinologic function at dose levels previously reported to be effective. Tests of DT $\lambda$ E at higher dose levels and the use of shorter sequence  $\beta$ -endorphin derivatives (for example,  $\beta$ -LPH<sub>66-77</sub>, DE $\lambda$ E) which also reportedly have neuroleptic-like activity are now under consideration.

Laboratory investigations in support of these clinical trials have recently focused on the synthesis and assay of enkephalins. Rat striated extracts yield several different molecular weight peptides which upon digestion can be shown to contain met-enkephalin. A membrane-bound peptidase has now been isolated from rat brain which forms met-enkephalin by cleavage of only medium weight peptides. Future studies will attempt to ascertain how this enzyme might affect the met-enkephalin pool size and thus possibly influence enkephalin mediated synaptic function. In a related study, high performance liquid chromatography (HPLC) and countercurrent chromatography have been systematically compared with respect to their ability to purify DE $\lambda$ E. The findings indicate that it is still preferable to perform countercurrent chromatography which provides quantitative recovery and then resort to HPLC for further purification.

## 6. Vasopressin Studies

Considerable evidence now implicates vasopressin in the regulation of mammalian learning and memory. In the rat, exogenously administered vasopressin facilitates various complex cognitive tasks including the consolidation and retrieval of learned responses. Uncontrolled clinical studies elsewhere suggested that vasopressin may improve aspects of attention and memory in normal individuals as well as those with certain amnesic disorders. During the past year a double blind, placebo controlled study of lysine vasopressin in 14 Alzheimer's disease patients has been completed. There was no consistent improvement in any measure of cognitive or memory function tested, despite use of drug treatment conditions previously reported



to be effective. One test of reaction time did, however, yield results consistent with an overall alerting action. Future plans include studies with higher doses of lysine vasopressin and possible use of longer acting and or less hormonally active analogues of vasopressin.

## 7. Neuroendocrine Studies

During the past year endocrinologic probes have attempted to further elucidate the extent of hypothalamic neuronal dysfunction in Huntington's chorea. Compared with control individuals, six Huntingtonian women were found to have a substantially elevated total daily secretion of growth hormone. Subsequent observations of an exaggerated growth hormone response to dopamine agonists, together with a blunted response to a dopamine antagonist, suggested that Huntington's disease might be associated with hyperfunction in the hypothalamic-pituitary dopamine system. On the other hand, preliminary results now indicate growth hormone hyperresponsivity occurs with pharmaceutical agents which selectively affect nondopaminergic systems. An explanation for these multiple neuroendocrinologic abnormalities is now being sought.

## 8. Cholecystokinin Studies

Recent laboratory studies have explored the ability of systemically administered cholecystokinin to influence central dopaminergic function. Cholecystokinin occurs in some dopamine-containing neurons and might thus affect dopamine mediated synaptic transmission. Like neuroleptic drugs which block dopamine receptors, cholecystokinin octapeptide (CCK-8) diminished apomorphine-induced stereotyped motor activity and reduced signaled-avoidance behavior in a dose-dependent manner. Moreover, CCK-8 significantly potentiated the ability of the neuroleptic, haloperidol, to impair avoidance responses in rats. In view of these encouraging results, an examination of the pharmacologic mechanisms by which peripherally administered CCK might influence central dopaminergic function is now beginning; preliminary data suggest that CCK-8 retards dopamine turnover in the median eminence.

## 9. Substance P Studies

Laboratory investigations of the behavioral effects of systemically administered substance P and several of its biologically active fragments have remained active in collaboration with the Department of Psychology, University of Colorado. Interest in this peptide derives from the strategic localization of substance P systems in relation to dopaminergic projections between the substantia nigra and corpus striatum. Preliminary results in mice suggest that low doses of substance P increase latency (improve memory) in passive avoidance learning paradigms, inhibit electrocortical shock-induced amnesia, potentiate amphetamine-induced stereotypy, and possibly exert an analgesic effect. This work will be expanded during the coming year.



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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF          INTRAMURAL RESEARCH PROJECT</b>	PROJECT NUMBER  Z01 NS 02236-06 ET																		
PERIOD COVERED October 1, 1980 to September 30, 1981																				
TITLE OF PROJECT (80 characters or less)  Diagnostic and Therapeutic Reevaluation of Patients with Intractable Epilepsy																				
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																				
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SUMMARY OF WORK (200 words or less - underline keywords) Despite recent advances in the therapy of <u>epilepsy</u> , many patients, especially those with <u>complex partial seizures</u> are incapacitated by their disorder. We have been investigating improvement of seizure control and reduction of medication side effects through the application of newly developed intensive monitoring techniques including simultaneous video-recording of <u>seizures</u> , long-term telemetering of EEGs and frequent determinations of <u>antiepileptic drug concentrations</u> . Patients with very long histories of uncontrolled seizures are admitted for a complete evaluation, including all basic neurologic studies and daily objective toxicity battery. <u>Video-recording</u> and long-term <u>telemetered EEGs</u> establish a seizure diagnosis, a concept which has not been adequately <u>emphasized</u> in the management of patients with <u>intractable seizure disorders</u> . Efforts are then made, based on this seizure diagnosis, to "tailor make" a regimen which is appropriate for each patient. This includes use of newer anti-epileptic medications which have decreased side effects in conjunction with <u>blood concentrations</u> which allow maximum therapeutic levels with minimal toxicity.																				

Project Description:Objectives:

The Clinical Epilepsy Section is undertaking a series of studies using new techniques of intensive monitoring of patients with intractable seizures in order to improve clinical control in many patients with refractory seizure problems, and can aid in the diagnosis of patients with disorders of unknown type such as psychogenic seizures. The fundamental method of therapy is medical, and modification of therapy will be dependent on collected information from all sources, including detailed history and examination, as well as routine and special laboratory studies, as indicated.

Methods:

Patients with intractable seizures are admitted to the Clinical Center according to the following criteria: 1) Primary consideration will be given to patients with complex partial seizures, although patients with other seizure types are also admitted. A limited number of patients with suspected psychogenic seizures are also admitted for study. 2) A history of uncontrolled seizures.

Simultaneous telemetered EEG and videotape recordings are made in six-hour periods from 0900 to 1500. Each patient has a minimum of one recording on every new regimen after drug steady-state level has been reached. This is compared with the baseline recording for evaluation of the new regimen.

Daily blood levels of antiepileptic drugs are determined by gas-liquid chromatography and by immunoassay in the pharmacology laboratory of the Epilepsy Branch.

After seizure frequency and type is characterized by the intensive monitoring techniques, correlation of this information is obtained with blood levels of antiepileptic drugs, and the therapeutic regimen is adjusted to obtain optimal seizure control. A small number of patients are considered for surgery, if medical therapy fails to control their seizures. Evaluation is conducted in cooperation with the Surgical Neurology Branch. Surgery has been performed in three cases in the past year.

A specific protocol has also been designed to investigate the etiologies of selected patients with epilepsy and progressive neurologic deterioration. This study, which involves a multidisciplinary team of investigators, is capable of analyzing neurologic, electroencephalographic, radiologic, pathologic (including brain biopsy when indicated), metabolic, and virologic data in an attempt to delineate some of the causes of seizure disorders.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

1) The Clinical Epilepsy Section has become a referral center for patients with intractable seizures, although only a very small number can be accepted into the program. Follow-up studies have been initiated and conducted on all patients who have undergone intensive monitoring to determine the current seizure frequency,

changes in social and rehabilitative status, and changes in the physical examination. At reevaluation after two years, 55% of the 74 patients with severe epilepsy still showed improved seizure control compared with preadmission frequency, 54% had reduced drug toxicity, and 38% had improved social adjustment. Sixty-nine percent showed improvement in at least one modality. Patients were able to take fewer medications than before admission.

2) Many intensive monitoring units report high rates of admission of patients with psychogenic seizures, showing the difficulty of making this diagnosis as an outpatient. Our assessment involved four major criteria: deviation of seizures from characteristics of known seizure types, absence of epileptiform activity in the ictal EEG, absence of slowing in the postictal EEG, and lack of increase in seizure frequency, with decreasing plasma concentrations of antiepileptic drugs. The clinical characteristics of psychogenic attacks were compared with generalized tonic-clonic and complex partial seizures. Although a one-year seizure-free period without medication may be the best criterion for psychogenic seizures, intensive monitoring greatly increases the accuracy of the diagnosis, using the criteria developed in this study.

3) It is important to distinguish between clinical seizure types in order to choose appropriate pharmacologic therapy. One of the most difficult differentiations is between complex partial and absence attacks. Psychogenic episodes are often mistakenly thought to be complex partial seizures. Electroencephalographic data are not always useful. Through a study of 163 video-taped seizures in 40 patients, clinical criteria were established which clearly define the characteristics of complex partial seizures, including onset, duration, ictal, and postictal phenomenology. Complex partial seizures may be distinguished from other seizure types or nonepileptic episodes on clinical grounds alone.

4) The question of the localization of the origin of generalized tonic-clonic seizures was also examined using videotapes. Only two of 36 generalized tonic-clonic seizures were of primary onset. Thirty-four were secondarily generalized, most frequently beginning with complex partial seizures. Adequate drug therapy reduced seizure duration. Primarily generalized tonic-clonic seizures are uncommon in patients with intractable epilepsy.

5) During the past year as part of an approved study of evoked responses of patients with complex partial seizures, normal subjects were evaluated to determine whether the visual evoked response is altered over the hemisphere opposite the nondominant eye. Among 25 normal subjects, the amplitude of the response of the right eye in the right eye dominant group was significantly higher than the responses in the left eye. A similar trend was noted with left eye dominant subjects, but the difference was not statistically significant. In addition, latencies of the P100 peak measured at  $O_2$  were shorter for the dominant eye. These findings demonstrate that the amplitude and latency disparities between dominant and nondominant eyes must be taken into account in any study involving VEPs, in particular studies designed for specific clinical diagnosis or for clinical investigation.

6) An important development in the evaluation of patients with intractable seizures is the new technique of positron emission tomography. In patients with intractable partial seizures, early studies have suggested that the local cerebral metabolic rate of glucose is reduced interictally in patients and increased in



patients during a seizure. Studies are now underway in patients under a variety of therapies with intractable multifocal seizures, partial seizures, and primary generalized seizures; these patients may also have significant abnormalities in the local cerebral metabolic rate of glucose. This finding may lead to improved localization for surgical therapy for the seizures in selected individuals. The data from the first nine patients studied with positron emission tomography are currently being evaluated. Four cases have shown good correlation between EEG and PET scan abnormalities. In some cases, the PET scan appeared to be able to localize an abnormality in the face of an equivocal EEG.

#### Proposed Course:

1. The Clinical Epilepsy Section continues to make clinical advances in intensive monitoring and therapy of patients with intractable epilepsy. Because of the remarkable heterogeneity of patients with intractable seizures, and because more information is gradually being obtained about the value of intensive monitoring as well as information on various seizure types, patients will continue to be accepted into this study. The program will continue to be active in technology transfer in intensive monitoring, both from technical and scientific standpoints.

2. The efforts of the Clinical Epilepsy Section will be directed toward utilization of the PET scan in evaluation of patients with uncontrolled seizures. Studies on patients with intractable partial seizures, primary generalized seizures, and multifocal seizures are currently underway; these studies are designed to determine whether changes in drug therapy or changes in the activity of the epileptiform electroencephalographic focus can be related to potential surgical therapy.

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SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02318-04 ET															
PERIOD COVERED October 1, 1980 to September 30, 1981																	
TITLE OF PROJECT (80 characters or less)  Clinical Pharmacology of Antiepileptic Drugs																	
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT																	
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SUMMARY OF WORK (200 words or less - underline keywords) The Clinical Epilepsy Section has been studying the <u>clinical pharmacology</u> of old and new <u>antiepileptic drugs</u> . Although many older medications have been marketed for years, there is still a great deal to be learned about the proper use of drugs which are already in our armamentarium. The Clinical Epilepsy Section is also actively involved in <u>clinical trials</u> of new antiepileptic drugs, such as sodium valproate, which was recently marketed in the United States, and progabide, which is completely new to the United States. This drug is being tested for epilepsy in eight European countries, and is presently being evaluated in man in the Clinical Epilepsy Section. The drug is a putative GABA agonist which may work by increasing inhibitory properties of nerve cells or synapses. Other studies underway include evaluation of carbamazepine absorption, differential phenytoin metabolism and phenytoin-carbamazepine interaction. The pharmacologic evaluation of these drugs is coupled with efficacy studies which are carried out by <u>intensive monitoring techniques</u> , videotape analysis of epileptic seizures with simultaneous telemetered EEG recording, and daily determination of antiepileptic drug levels.																	

Project Description:Objectives:

This project includes a large number of independent pharmacologic studies in the investigation of the clinical pharmacology of antiepileptic drugs. The object of each study is different, but each may include obtaining such pharmacologic data as (1) single dose plasma half-lives, (2) relative plasma levels of parent drugs and metabolites with chronic administration, (3) relationships of the efficacy of parent drugs and/or metabolite to plasma levels, (4) efficacy of various compounds against different seizure types as correlated with intensive monitoring techniques, (5) evaluation of efficacy, toxicity, and pharmacology of new antiepileptic agents, such as progabide, (6) measurement of rate of biotransformation of various antiepileptic medications, and (7) determination of the clinical consequences of withdrawal of antiepileptic drugs.

Methods:

Patients with uncontrolled seizures, especially complex partial seizures, are accepted for study. Such patients usually have a detailed seizure calendar available prior to entering the study, and enter a week-long period of baseline determination of seizure frequency and blood levels of antiepileptic drugs while in the hospital. Each pharmacologic protocol varies, but all require modification of the antiepileptic regimen and addition of the medication under study. This may be done in single dose or chronic administration studies depending upon the particular protocol in question. As a rule, plasma levels are drawn at least daily, and on occasion, much more frequently for specific studies. Following the completion of the pharmacologic protocol, the patient is placed on a regimen which is best suited for the seizure type which has been identified by videotape/telemetered EEG analysis. This regimen is stabilized prior to discharge of the patient.

Normal volunteers participate in pharmacologic studies of new drugs which require that no other drugs be taken. This allows increased knowledge of the action of these new drugs in the absence of interfering factors such as medications necessary to control seizures; it is the baseline for future studies in patients with epilepsy.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

1) A new drug for epilepsy is being evaluated in eight European countries, but is being tested in the U.S. for the first time in the Clinical Epilepsy Section. The drug is called progabide, and has been investigated abroad in several hundred patients with epilepsy with encouraging results. A new micronized formulation has necessitated reevaluation of the pharmacologic properties of the drug, and this is being accomplished at present. Studies are being performed on normal volunteers with single doses, measuring the elimination half-life of the drug and its metabolites. These data will permit further studies in patients with seizures. Pilot studies in epilepsy will therefore follow these pharmacologic investigations in normal volunteers. The significance of finding a new, efficacious drug for the control of epilepsy is quite remarkable, considering that approximately two million Americans have the disorder. A new drug may control only a small

percentage of the afflicted persons, yet still have a remarkable impact on thousands of patients.

2) Although interest has recently developed on the effect of food on absorption of a wide variety of medications, little evidence has thus far accumulated on the effect of food on antiepileptic drugs. In the studies conducted by the Clinical Epilepsy Section the effect of taking medication with food and without food has been analyzed in eleven patients. Steady-state levels obtained over two two-week periods have been analyzed. Steady-state levels do not appear to change, but food does appear to affect the rate of absorption of carbamazepine in some patients. These preliminary findings suggest that food may be an important factor in the absorption of medications in individual patients, and that careful drug administration may greatly influence the total dose of drug that a patient can tolerate. The effect of food may have profound importance for dosage schedules, instructions to patients, and maximum amount of medication which is tolerated. This is especially important for severely affected patients because of the need for maximal doses of medications to obtain optimal control.

3) Recognition of their subtle but significant toxicity has made the need for sedative-hypnotic antiepileptic drugs less certain, but their removal is often thought to be difficult and dangerous. Barbiturates and benzodiazepines were completely withdrawn from 78 patients with intractable epilepsy (48 inpatients and 30 outpatients). After 6 months of outpatient follow-up, 69 patients were still on a nonsedative regimen: 56 showed improvement in either toxicity or seizure control or both. Nonsedative drugs were adjusted to provide optimal seizure control. Larger doses of more effective medications were tolerated once sedative-hypnotic drugs were withdrawn. Significant social and behavioral improvements also occurred in many patients. Withdrawal was well tolerated, and status epilepticus did not occur. This study shows that sedative drugs are not necessary for optimal seizure control, even in intractable epilepsy, and that they may be safely withdrawn.

4) Most of the drugs currently employed in the treatment of epileptic disorders have a relatively narrow therapeutic ratio and when used in conjunction with other antiepileptics or other drugs, they frequently become involved in drug-drug interactions. The propensity of these agents for drug-drug interaction complicates effective and safe management of seizures. Clinical observations have shown a rise in phenobarbital levels in patients taking primidone when phenytoin was added to their regimens. In some cases, sedation and other phenobarbital toxicity was observed. A pharmacologic study was performed to analyze this effect. Drug metabolites were measured when patients were taking primidone and phenytoin, or primidone alone. The phenobarbital/phenylethylmalonamide (PEMA) ratio was elevated, but PEMA levels were not decreased on the combination regimen. Phenytoin was found to inhibit phenobarbital metabolism directly, rather than indirectly by affecting primidone degradation. Valproic acid, a relatively new antiepileptic drug, has been shown to increase phenobarbital levels in patients taking both drugs. Clinical toxicity has occurred in some cases. An initial study using a stable isotope labelled phenobarbital showed increased phenobarbital half-life and urinary excretion of unmetabolized drug. Phenobarbital plasma clearance and elimination rate constant were decreased. These findings suggested that valproic acid inhibited phenobarbital metabolism. In order to elucidate the



mechanism of this effect, the influence of valproic acid on acetaminophen degradation was studied. This drug is metabolized through glucuronidation, while for phenobarbital, both glucuronidation and parahydroxylation occur. Valproic acid did not affect acetaminophen levels or elimination. Thus, its effect on phenobarbital is likely to be an inhibition of hydroxylation rather than glucuronidation.

5) The Clinical Epilepsy Section will shortly begin studies on differential phenytoin metabolism. Different racial groups and nationalities may have different pharmacokinetic rates with a wide assortment of medications. Since phenytoin is used internationally as an antiepileptic drug, the determination of differences in the pharmacokinetics of phenytoin among different nationalities is quite important. The Clinical Epilepsy Section is initiating studies with stable labelled isotopes which will allow careful and accurate identification of the metabolism of phenytoin in patients without having to withdraw the patients from this medicine. The NIH study is a pilot study for this protocol, using the stable labelled isotope to evaluate the adequacy of the proposed protocol; an international study is planned to follow.

6) The effect of carbamazepine on the pharmacokinetic and metabolic parameters of phenytoin will be determined. In this study, oral phenytoin will be given to epileptic patients with uncontrolled seizures. A dose of heavy-labelled phenytoin will be given to determine the pharmacokinetic parameters of phenytoin alone. Following this, carbamazepine will be added to the regimen and another pulse dose of labelled phenytoin administered. The differences will be analyzed to determine the interaction between carbamazepine and phenytoin. These results will be useful in understanding the mechanisms by which carbamazepine interacts and changes the plasma levels of phenytoin when both drugs are given in combination.

#### Proposed Course:

1. Future studies will attempt to elucidate further such variables as drug absorption, distribution, metabolism, excretion, interaction, efficacy, toxicity, and withdrawal.

2. A planned project for the Clinical Epilepsy Section will be directed toward the study of differential phenytoin metabolism in different races. A study for an international protocol is planned for the NIH using a stable labelled isotope to evaluate the adequacy of a pilot protocol.

3. Potential new drugs, such as progabide, will be studied both for their efficacy and their pharmacology.

#### Publications:

Kapetanovic I, Kupferberg HJ, Porter RJ, Penry JK: Valproic acid-phenobarbital interaction: a systematic study using stable isotopically labelled phenobarbital in an epileptic patient. In Johannessen SI, Morselli PL, Pippenger CE, Richens A, Schmidt D, Meinardi H (Eds): Antiepileptic Therapy: Advances in Drug Monitoring. Raven Press, New York, 1980, pp 373-380.

Theodore WH, Porter RJ: Removal of sedative antiepileptic drugs from the regimens of patients with intractable epilepsy. Trans. Am. Neurol. Assoc. 1981 (in press).



Porter RJ, Schulman EA, Penry JK: Phenytoin monotherapy in intractable epilepsy: In Canger R, Angeleri F, Penry JK (Eds): Advances in Epileptology: XIth Epilepsy International Symposium. Raven Press, New York, 1980, pp 419-422.

Kapetanovic IM, Kupferberg HJ, Theodore W, Porter RJ: Lack of effect of valproate and paracetamol (acetaminophen) disposition in epileptic patients. Br. J. Clin. Pharmacol. 11:391-393, 1981.

Porter RJ: Pharmacokinetic basis of intermittent and chronic anticonvulsant drug therapy in febrile seizures. In Nelson KB, Ellenberg JH (Eds): Febrile Seizures: Long Term Management of Children with Fever-Associated Seizures. Raven Press, New York, 1981 (in press).

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Porter RJ, Kupferberg HJ: Other succinimides: methsuximide and phensuximide. In Woodbury DM, Penry JK, Pippenger CE (Eds): Antiepileptic Drugs, Second Edition. Raven Press, New York, 1981 (in press).

Kapetanovic IM, Kupferberg HJ: Gas chromatographic and gas chromatographic-mass spectrometric determination of p-hydroxyphenobarbital extracted from plasma, urine, and hepatic microsomes. J. Pharm. Sci. 1981 (in press).

Bius DL, Yonekawa WD, Kupferberg HJ, Cantor F, Dudley KH: Gas chromatographic-mass spectrometric studies on the metabolic fate of ethotoin in man. Drug Metab. Dispos. 8:223-229, 1980.

Kapetanovic I, Kupferberg HJ: Stable isotope methodology and gas chromatography-mass spectrometry in a pharmacokinetic study of phenobarbital. Biomed. Mass Spectrom. 7:47-52, 1980.

C.T. No. 76N344  
C.T. No. 77N92  
C.T. No. 78N171  
C.T. No. 79N04

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  <div style="text-align: center; font-weight: bold;">Z01 NS 02263-05 ET</div>
PERIOD COVERED      October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less) Biochemical and Pharmacological Studies of Dopamine Receptors		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	J. W. Keabian	Chief, Biochemical Neuropharmacology Section      ETB NINCDS
Other:	T. E. Cote H. Stoof K. Tsuruta E. Frey R. Eskay R. Long	Staff Fellow      ETB NINCDS Visiting Associate      ETB NINCDS Visiting Fellow      ETB NINCDS Staff Fellow      ETB NINCDS Senior Staff Fellow      LCS NIMH Biologist      LCS NIMH
COOPERATING UNITS (if any)		
Laboratory of Clinical Science, NIMH		
LAB/BRANCH Experimental Therapeutics Branch		
SECTION Biochemical Neuropharmacology Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Md. 20205		
TOTAL MANYEARS: 6.3	PROFESSIONAL: 5.3	OTHER: 1.0
CHECK APPROPRIATE BOX(ES)		
<input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER		
<input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)		
<p>           This project identifies and characterizes biochemical mechanisms for <u>dopamine receptors</u>. An understanding of the biochemical phenomena contributing to the activity of dopamine receptors will identify possible mechanisms of action for drugs used to treat <u>Parkinson's disease</u>, <u>endocrine disorders</u>, and <u>psychiatric disorders</u>. Among the topics studied in the current fiscal year are: investigation of the site of action of guanyl nucleotides in regulating beta-adrenergic stimulation and dopaminergic inhibition of adenylate cyclase activity in the intermediate lobe of the rat pituitary gland; 2) identification of specific D-2 receptor agonists; 3) demonstration that the D-1 receptor stimulates and D-2 receptor inhibits cAMP formation in the neostriatum.         </p>		

Project Description:Objectives:

- 1) Study the dopamine receptor in the intermediate lobe of the rat pituitary gland.
- 2) Identify drugs which selectively interact with the D-2 dopamine receptor in the intermediate lobe of the rat pituitary gland.
- 3) Apply information about the biochemistry and pharmacology of dopaminergic neurotransmission in the intermediate lobe to neural tissues receiving dopaminergic innervation.

Methods:

The intermediate lobe (IL) of the rat pituitary gland contains a beta-adrenoceptor. The presence of this receptor is inferred from observations that beta-adrenergic agonists enhance the release of alpha-melanocyte stimulating hormone ( $\alpha$ MSH), enhance the formation of cyclic AMP, stimulate adenylate cyclase activity, and can interact with specific binding sites. The linkage of the beta-adrenoceptor to adenylate cyclase is organized in a manner similar to other tissues. In the studies performed during FY '81, IL tissue was treated with cholera toxin in order to maximally activate adenylate cyclase activity by stimulating the guanyl nucleotide regulatory site interposed between the  $\beta$ -adrenoceptor and adenylate cyclase.

In studies of neural tissue, slices of neostriatal tissue were superfused in a 16-chamber superfusion apparatus. The tissue was exposed to drugs and the amount of cyclic AMP released into the medium was estimated by radioimmunoassay.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

- 1) During FY '81, the stimulatory effect of guanyl nucleotides upon IL adenylate cyclase was characterized. Guanosine 5'-triphosphate (GTP) participates in receptor-mediated stimulation of adenylate cyclase in many different cells. GTP is believed to interact with a stimulatory guanyl nucleotide component (designated by Rodbell as  $N_s$ ) and thereby enhance adenylate cyclase activity. The involvement of a stimulatory guanyl nucleotide component in the activation of adenylate cyclase activity in the IL of the rat pituitary gland was indicated by the following observations: 1. GTP is required for a  $\beta$ -adrenergic agonist to enhance adenylate cyclase activity; 2. under appropriate conditions GMPP(NH)P and sodium fluoride stimulate adenylate cyclase activity in the absence of a  $\beta$ -agonist; 3. cholera toxin confers sensitivity to GTP upon adenylate cyclase in the absence of a  $\beta$ -agonist; and 4. GTP decreases the affinity of the IL  $\beta$ -adrenoceptor for agonists.

When interpreted within the framework provided by Rodbell's model of stimulatory receptors, the data obtained in FY '81 suggest that the  $\beta$ -adrenoceptor in the IL is coupled to adenylate cyclase by  $N_s$ . Stimulation of the  $\beta$ -adrenoceptor in the IL alters the properties of  $N_s$  so that intra-cellular GTP stimulates adenylate

cyclase activity. The enhanced synthesis of cAMP initiates a series of unknown events ultimately expressed as an increased release of  $\alpha$ MSH.

2) In FY '81, the role of GTP in the functioning of the D-2 receptor in the IL was investigated. In order to study the involvement of GTP in the dopaminergic inhibition of adenylate cyclase activity, IL tissue was treated with cholera toxin. Cholera toxin catalyzes the ADP-ribosylation of  $N_i$ ; this modification is believed to inactivate a GTPase associated with  $N_i$ , and thus induce a persistent activation of adenylate cyclase activity in the presence of GTP. IL tissue treated with cholera toxin has elevated adenylate cyclase activity and responds poorly to  $\beta$ -adrenergic stimulation. However, cholera toxin treatment appears not to affect functioning of the dopamine receptor. Thus, the molar potency of either dopamine or apomorphine as an inhibitor of adenylate cyclase activity was not altered by cholera toxin treatment. Furthermore, although cholera toxin treatment abolished the ability of isoproterenol to stimulate cAMP formation or  $\alpha$ MSH release by melanotrophs, apomorphine effectively diminished both cAMP formation and  $\alpha$ MSH release from these cells. Because cholera toxin treatment increases adenylate cyclase activity in the absence of any  $\beta$ -adrenergic agonists, the inhibitory effects of guanyl nucleotides and dopaminergic agonists upon enzyme activity could be more easily studied in cholera toxin-treated tissue than in fresh tissue.

Dopaminergic agonists inhibited adenylate cyclase activity in cholera toxin-treated IL tissue only in the presence of the guanyl nucleotide, GTP. Dopaminergic antagonists blocked this inhibition thereby suggesting that the dopaminergic agonists interacted with a dopamine receptor.

In the absence of a dopaminergic agonist, nonhydrolyzable analogues of GTP inhibited adenylate cyclase activity; GTP reversed the inhibitory effect of GMPP(NH)P. The potency of GTP depended upon the concentration of GMPP(NH)P, thus suggesting a kinetic competition between the two compounds. Assuming a kinetic competition between GTP and GMPP(NH)P, the affinity of GTP for the active site on the inhibitory guanyl nucleotide component was calculated to be 0.6  $\mu$ M.

Dopaminergic antagonists did not prevent the inhibitory effect of GTP analogues. Thus, the GTP analogues appear not to interact directly with the IL dopamine receptor. However, stimulation of the dopamine receptor in some way alters the properties of the inhibitory guanyl nucleotide component. Apomorphine, a dopaminergic agonist, abolished the ability of GTP to reverse the inhibition by GMPP(NH)P; this effect of apomorphine was prevented by fluphenazine, a dopaminergic antagonist.

These data, when interpreted within the framework provided by Rodbell's model of inhibitory receptors, suggest that the D-2 receptor in the IL is coupled to adenylate cyclase by  $N_i$ . Assuming the existence of  $N_i$ , we adopted the following "working hypothesis" to account for the effects of dopaminergic agonists and guanyl nucleotides on adenylate cyclase activity. Occupancy by an agonist (i.e. stimulation) of the D-2 receptor in some way alters the properties of  $N_i$  so that GTP can interact with  $N_i$  and inhibit adenylate cyclase activity. When adenylate cyclase activity is increased by stimulation of the  $\beta$ -adrenoceptor or treatment with cholera toxin, the



inhibitory dopaminergic effect is more pronounced. In the absence of a dopaminergic agonist, GMPP(NH)P interacts with  $N_1$  and, in some way, alters  $N_1$  so as to inhibit adenylate cyclase activity. This assumption accounts for the ability of GTP to reverse the inhibitory effect of GMPP(NH)P. Our data suggest that stimulation of the D-2 receptor does not alter the affinity of GTP for  $N_1$ . Fluoride also appears to affect  $N_1$  but at a site distinct from the guanyl nucleotide regulatory site; thus, the inhibitory effect of fluoride is not reversed by GTP.

In summary, GTP represents a potential endogenous constituent of the melanotroph which can either increase or decrease the synthesis of cAMP depending on the occupancy of the  $\beta$ -adrenoceptor or the dopamine receptor. Changes in the intracellular level of cAMP regulate the release of  $\alpha$ MSH via an unknown mechanism (s). The ability of cholera toxin to increase cAMP formation and increase the efflux of  $\alpha$ MSH is in accord with the hypothesis that GTP,  $N_1$ , and cAMP are important constituents of the IL involved in the  $\beta$ -adrenergic enhancement of  $\alpha$ MSH release. The ability of a dopaminergic agonist to decrease cAMP formation and decrease the efflux of  $\alpha$ MSH in cholera toxin-treated IL tissue is in accord with the hypothesis that GTP,  $N_1$ , and cAMP participate in the dopaminergic inhibition of  $\alpha$ MSH release.

3) According to the classification schema of dopamine receptors formulated in ETB, there are two general categories of dopamine receptor designated as D-1 and D-2 receptors. In order to substantiate the validity of the classification schema, it was desirable to identify compounds which selectively interacted with the two categories of dopamine receptor. In FY '81, LY-141865 (trans-( $\pm$ )-4,4a,5,6,7,8,8a,9-octahydro-5-propyl-2H pyrazolo-(3,4-g)quinoline) was shown to discriminate between the D-1 and the D-2 receptor. LY-141865 did not interact with the D-1 receptor. LY-141865 did not stimulate the D-1 receptor in the carp retina; and unlike lergotril and lisuride, LY-141865 did not block this D-1 receptor. LY-141865 was shown to stimulate the D-2 receptor in the rat IL because: 1. LY-141865 mimicked the ability of dopamine and dopaminergic agonists to decrease basal of L-isoproterenol stimulated release of  $\alpha$ MSH; 2. LY-141865 mimicked the inhibitory actions of dopamine and dopaminergic agonists upon adenylate cyclase activity in homogenates of fresh or cholera toxin-treated tissue; and 3. the inhibitory effect of LY-141865 upon adenylate cyclase activity was reversed by dopamine antagonists, including the selective D-2 antagonist (-) sulpiride. The availability of drugs selectively stimulating either the D-1 receptor (SKF 38393A) or the D-2 receptor (e.g. LY-141865) may facilitate pharmacological analysis of the physiological responses to dopamine. Furthermore, because LY-141865 penetrates the blood-brain barrier following its systemic administration, it may be of use in the treatment of Parkinsonism. It was especially gratifying to have the report of the Section's work with LY-141865 accepted by Nature as submitted.

4) During FY '81, the possibility that dopamine and drugs stimulating the pituitary D-2 dopamine receptor might inhibit the formation of cAMP in the neostriatum was investigated.

Previously, stimulation by dopamine of either adenylate cyclase activity in cell-free homogenates of neostriatal tissue or cAMP accumulation in blocks of neostriatal tissue had been used as biochemical signs of D-1 dopamine receptor stimulation. However, during FY '81, the efflux of cAMP from blocks of rat



neostriatum was shown to provide an additional biochemical sign of the stimulation of the D-1 receptor in this tissue. The ability of SKF 38393 to increase the efflux of cAMP from neostriatal blocks is in accord with previous demonstrations that this compound stimulates striatal dopamine-sensitive adenylate cyclase. The doses of SKF 38393 half-maximally stimulating cAMP efflux and half-maximally activating dopamine-sensitive cyclase are approximately the same ( $2 \times 10^{-7}$  M and  $10^{-7}$  M, respectively). However, the maximal effect of SKF 38393 upon cAMP efflux (a 3.5-fold enhancement) was greater than the maximal effect of the drug upon either adenylate cyclase activity (a 1.7-fold enhancement) or the cAMP content of the neostriatal blocks (a 1.6-fold enhancement). The possibility that the SKF 38393-stimulated efflux of cAMP occurred as a consequence of stimulation of a D-1 receptor was supported by observations that this drug-stimulated efflux of cAMP is antagonized by fluphenazine but not by (-)-sulpiride. The stimulation of cAMP efflux by either dopamine or apomorphine (in the presence of (-)-sulpiride) is in accord with previous demonstrations that these agonists enhance striatal adenylate cyclase activity. The failure of LY-141865 to increase the efflux of cAMP is consistent with the previous demonstration that this agonist does not interact with the D-1 dopamine receptor (see section 3).

The results obtained in FY '81 suggest the existence in the neostriatum of two dopamine receptors regulating the efflux of cAMP. One dopamine receptor is the D-1 dopamine receptor; stimulation of this receptor enhances the synthesis and efflux of cAMP. We propose that the second dopamine receptor resembles the D-2 dopamine receptor in the pituitary gland. The biochemical consequences of stimulation of the pituitary D-2 dopamine receptor and the second striatal dopamine receptor are similar. Stimulation of the pituitary dopamine receptor decreases the synthesis of cAMP; the present results demonstrate that stimulation of the second striatal dopamine receptor inhibits the efflux (and by inference the synthesis) of cAMP occurring as a consequence of stimulation of the D-1 receptor. Dopamine, apomorphine and LY-141865 are agonists upon both the pituitary D-2 dopamine receptor and the second neostriatal dopamine receptor; (-)-sulpiride is more potent than is (+)-sulpiride as an antagonist upon either D-2 dopamine receptor. According to our interpretation of the present data, dopamine interacts with both types of receptor in the neostriatum, simultaneously stimulating and inhibiting the synthesis and efflux of cAMP. (-)-Sulpiride potentiates the dopamine-stimulated efflux of cAMP by blocking the second dopamine receptor and removing the inhibitory constraint upon the synthesis and efflux of cAMP. Similarly, apomorphine stimulates both the D-1 and the second striatal dopamine receptor; (-)-sulpiride antagonizes the interaction between apomorphine and the D-1 receptor to be expressed as an enhanced efflux of cAMP. The SKF 38393-stimulated efflux of cAMP is not potentiated by (-)-sulpiride because at a concentration of  $1 \mu$ M SKF 38393 specifically stimulates the D-1 dopamine receptor. However, the SKF-38393-stimulated efflux of cAMP is diminished by LY-141865 as a consequence of the interaction between LY-141865 and the second, pituitary-like D-2 dopamine receptor. Accordingly, this effect of LY-141865 is blocked by (-)-sulpiride.

#### Proposed Course:

In the coming year, the Section will continue to investigate the intermediate lobe of the rat pituitary gland. Using high specific activity spiroperidol,

binding studies will be performed to identify the dopamine receptor in the rat IL. The properties of the spiroperidol binding site will be compared to the properties of the dopamine receptor regulating  $\alpha$ MSH release. Such a comparison will permit insight into the problem of how many dopamine receptors must be stimulated to either inhibit adenylate cyclase activity in a cell free homogenate or to inhibit the release of  $\alpha$ MSH.

In the coming year attention will be directed towards the regulation of  $\alpha$ MSH synthesis in the rat IL. The ability of  $\beta$ -adrenergic agonists and D-2 dopaminergic agonists to affect the synthesis of  $\alpha$ MSH will be investigated. The role of these investigations will be to determine if neurotransmitters can affect the expression of genetic information.

The knowledge obtained from the intermediate lobe about the biochemical basis of dopamine's action will be applied to investigations of the regions of the brain receiving dopaminergic innervation. The regulation of the release of acetylcholine from the neostriatum and the development of supersensitivity of the D-1 receptor following denervation are two experimental areas which may be amenable to experimental investigation with the resources available in the coming year.

#### Publications:

- Kebabian, J.W. and Zatz, M.: Adaptive properties of adrenoceptors. In Cotman, C.W., Poste, G., and Nicolson, G.L. (Eds.): Cell Surface Reviews the Cell Surface and Neuronal Function. Amsterdam, Elsevier, 1980, pp. 303-349.
- Munemura, M., Cote, T.E., Tsuruta, K., Eskay, R.L., and Kebabian, J.W.: The dopamine receptor in the intermediate lobe of the rat pituitary gland: pharmacological characterization. Endocrinology 107: 1676-1683, 1980.
- Kebabian, J.W., and Cote, T.E.: Dopamine receptors and cyclic AMP: a decade of progress. TIPS 2: 69-71, 1981.
- Kebabian, J.W., Tsuruta, K., Cote, T.E., and Grewe, C.W.: The activity of substituted benzamides in biochemical models of dopamine receptors. In Stanley, M. and Rothrosen, J. (Eds.): The Substituted Benzamides. New York, Raven Press, 1981. In press.
- Tsuruta, K., Frey, E.A., Grewe, C.W., Cote, T.E., Eskay, R.L., and Kebabian, J.W.: Evidence that LY-141865 specifically stimulates the D-2 dopamine receptor. Nature, 1981. In press.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02139-07 ET
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less)  Pharmacology and Physiology of Central Neurotransmitters		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  PI: J.R. Walters      Head, Physiological Neuropharmacology Section, ETB, NINCDS  Others: B.L. Waszczak      Staff Fellow      ETB NINCDS D. Bergstrom      Staff Fellow      ETB NINCDS R. Ross      Staff Fellow      NIGMS D. Jackson      Guest Worker      ETB NINCDS L. Miller      Guest Worker      NHLBI		
COOPERATING UNITS (if any)  None		
LAB/BRANCH Experimental Therapeutics Branch		
SECTION Physiological Neuropharmacology		
INSTITUTE AND LOCATION NINCDS,NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 4.7	PROFESSIONAL: 3.5	OTHER: 1.2
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The purpose of this project is to develop an understanding of the role of specific neurotransmitters in basal ganglia function, with the goal of developing improved strategies for <u>pharmacological treatment of neurological disorders</u> . Topics currently under investigation include (1) the ways in which <u>dopamine and dopamine agonists</u> may affect <u>neuronal activity in the globus pallidus and the substantia nigra</u> ; (2) changes in the dopaminergic system induced by <u>chronic dopamine agonist treatment</u> and (3) effects of <u>GABA, GABA agonists</u> , and drugs, such as the <u>benzodiazepines</u> which modulate GABA's effects on the activity of identified regions of the <u>basal ganglia and substantia nigra</u> .		

Project Description:Objectives:

This project involves investigation of the role of specific neurotransmitters in regulating neuronal activity in extrapyramidal systems. The long range goal is to establish improved pharmacological treatment to compensate for apparent neuronal degeneration and dysfunctions which occur in a variety of neurological disorders involving the basal ganglia and substantia nigra regions. The short range goal is to develop a better understanding of how specific neurotransmitter systems, especially those using dopamine and GABA as neurotransmitters, and the agonists of these neurotransmitters, affect information processing in the basal ganglia.

Methods:

These studies utilize (a) determination of single-unit activity of identified brain cells in anesthetized rats, some with neurotoxin-induced lesions of specific brain regions, (b) determination of single-unit activity in identified brain regions in rats which are locally anesthetized, paralyzed and artificially re-spined, (c) investigation of effects of microiontophoresed drugs or neurotransmitter substances on single-unit activity, (d) identification of cells by antidromic stimulation and (e) estimation of brain lesion or drug-induced changes in levels of dopamine, dopamine metabolites and glutamic acid decarboxylase activity.

Major Findings and Significance to Biomedical Research and the Program of the Institute:

(1) Physiological effects of dopamine and dopamine agonists in the basal ganglia.

To gain insight into how dopamine and the dopamine agonists affect information processing in the basal ganglia, we have been examining the effects of these agents at sites where information funnels out of the basal ganglia; the pars reticulata of the substantia nigra and the globus pallidus. We have also continued our investigations of how dopamine agonists affect the activity of the dopamine neurons themselves.

(a) Substantia Nigra Pars Reticulata Studies

The pars reticulata region of the substantia nigra is ventral to the dopaminergic pars compacta region. The major input to the pars reticulata neurons is from the striatum. The reticulata cells project to the superior colliculus where they are thought to play a role in the regulation of eye movements; to the VA and the VL of the thalamus, an area which in turn projects to the motor cortex; and downstream to the reticulata formation. Because the pars reticulata cells receive a projection from the striatum, they are in a position to be indirectly affected by the action of dopamine and dopamine agonists in the striatum. In addition, it has been postulated that dopamine neurons may release dopamine from their dendrites in the pars reticulata and, therefore, may also directly affect the activity of the reticulata neurons. We have begun to investigate these possible influences of dopamine on rat reticulata cell activity by studying the response of the reticulata cells to iontophoresed dopamine. In addition, we have been investigating the



possibility that dopamine might be able to affect neuronal activity in the pars reticulata by modulating the effects of other neurotransmitters on these cells. It has been increasingly appreciated recently that other monoamines, such as norepinephrine and serotonin, affect neuronal activity in some areas by such a modulatory action.

We have found that iontophoresis of dopamine causes increases in reticulata cell activity to an average of 27% over baseline. More significantly, dopamine consistently and markedly attenuated the inhibitory responses of cells to GABA. The degree of this attenuation was not correlated with, nor dependent upon, the increase in firing. Both the absolute inhibition, in numbers of spikes, as well as the percent inhibition by GABA were significantly reduced by applied dopamine. In contrast to its attenuation of GABA responses, dopamine did not consistently alter responses of cells to iontophoresed glycine.

To assess the specificity of these actions of dopamine on reticulata neurons, studies were conducted in which an equimolar solution of norepinephrine was substituted for dopamine. While norepinephrine caused similar average increases in firing to 30% over baseline, it did not consistently or significantly alter, overall cells tested, the absolute amount of inhibition elicited either by GABA or glycine.

To determine whether cells in the pars reticulata projecting to one of the major projection areas of this nucleus might have properties which distinguished them from cells projecting to other areas, we identified by antidromic stimulation those cells projecting to the ipsilateral ventral medial nucleus of the thalamus. The responses of these nigrothalamic neurons to dopamine and the other neurotransmitters studied was not significantly different from those of cells which were not determined to be projecting to the thalamus.

These results indicate that dopamine, released from the dendrites within the nigra, and systemically administered dopamine agonists, could potentially modulate the actions of GABA on substantia nigra pars reticulata neurons projecting to the ventromedial nucleus of the thalamus and to other areas. The increased firing elicited by dopamine alone may reflect either the neurotransmitter's direct excitatory action and/or its ability to modulate inhibitory influences provided by the GABAergic inputs these cells receive. This demonstration of dopamine's ability to modulate GABA's inhibitory effects on neuronal activity provides the basis for hypothesizing a novel and potentially very significant mechanism through which dopamine and dopamine agonists may act to alter information-processing in the basal ganglia.

#### (b) Globus Pallidus Studies

We have also been interested in determining how dopaminergic drugs affect neuronal activity in the globus pallidus (external pallidus) of the rat. There are several sites where dopamine can act to influence activity in the external segment of the globus pallidus. The external pallidus, like the pars reticulata of the substantia nigra, receives a major input from the striatum, which is, in turn, heavily innervated by dopamine neurons. In addition, there is some evidence that dopamine cells may send a direct projection to the globus pallidus and may



also communicate with the external pallidus via the subthalamic nucleus which, some have reported, receives a dopaminergic input.

To study the effects of increased dopamine release on the tonic activity of pallidal neurons, the effects of systemic administration of d-amphetamine on pallidal activity has been assessed. We found that systemic administration of d-amphetamine causes significant increases in the unit activity of spontaneously firing neurons in this area. This action seems related to the ability of d-amphetamine to release dopamine since l-amphetamine, which produces similar effects on norepinephrine release but smaller effects on dopamine release caused only a slight excitation of pallidal neurons. The serotonin uptake inhibitor fluoxetine, produced only varied changes in firing, while minor effects were also observed after systemic administration of desmethylinipramine and clonidine. The results suggest that amphetamine-induced release of dopamine is associated with a marked stimulation of firing rates of spontaneously active globus pallidus cells in gallamine-paralyzed rats.

In considering the actions of dopamine agonists in the basal ganglia, one must take into consideration not only the action of these drugs at sites which are normally affected by released dopamine but also the possibility of action at additional dopamine receptor sites where the existence of direct dopaminergic innervation or functional release is not yet clearly established. These include dopamine autoreceptors on the dopamine cell bodies and terminals, dopamine receptors on striatonigral and corticostriatal axons and on cells in the pars reticulata of the substantia nigra and in the globus pallidus. Moreover, these receptors and those which are more uncontroversially postsynaptic dopamine receptors may vary in their affinity for different dopamine agonists and antagonists and in their function. How the actions of dopamine agonists at these different dopamine receptor subtypes and in different anatomical regions summate to affect information processing in the basal ganglia is not clear. It has been hypothesized that some of the paradoxical or biphasic behavioral effects of dopamine agonists, such as apomorphine, reflect differences in the relative sensitivities of different dopamine receptors. Low doses of apomorphine appear to act preferentially at autoreceptors on dopamine neurons to decrease dopamine cell activity and dopamine release, causing a net decrease in postsynaptic dopamine receptor stimulation, whereas larger doses of apomorphine stimulate postsynaptic striatal dopamine receptors.

To gain insight into the net effects of dopamine agonists in the basal ganglia, we have investigated the effects of systemic administration of apomorphine on the tonic activity of neurons in the external pallidum. It seemed possible that the tonic activity of globus pallidus neurons would be influenced by the level of postsynaptic dopamine receptor stimulation and might reflect the hypothesized biphasic effects of dopamine agonists interacting with the various subcategories of pre- and postsynaptic dopamine receptors in the basal ganglia and substantia nigra. Like amphetamine, apomorphine, in doses of .08 to 1.0 mg/kg, markedly enhanced the firing of spontaneously active pallidal neurons. However, low doses (5, 20  $\mu$ g/kg) which preferentially stimulate presynaptic dopamine receptors did not cause effects opposite to those observed with larger doses in 96% of the cells monitored. Thus, the paradoxical effects of low doses of dopamine agonists are not reflected in alterations in the tonic activity of cells in the globus pallidus.

On the other hand, additional observations made during these studies may provide new clues about some of the paradoxical effects of these drugs. Our findings show that the expression and magnitude of the excitation induced by apomorphine are influenced by the schedule of drug administration. When a nonexcitatory, small dose of apomorphine is given before a dose which, by itself, causes a maximal stimulation of pallidal activity, the first dose appears to have a priming effect, altering or setting the systems response, so that additional injections of apomorphine have little further effect. While the mechanisms or sites of drug action responsible for this phenomenon are not known, the results suggest a sufficient number of agonist molecules must reach the dopamine receptors in a short time interval for a maximal response to be elicited. Similarly, one might hypothesize that when fewer receptors are occupied, a change in the conformational state of the receptor complex is induced, a weaker response is elicited, and the receptor complex is rendered less sensitive to additional agonist stimulation. These results suggest that the method of apomorphine administration should be taken into consideration when the effects of this drug on brain metabolism, animal behavior, and possibly, therapeutic potential are being investigated. The results also raise the possibility that some of the useful paradoxical effects of dopamine agonists in hyperkinetic disorders may be related to the "priming effects" of low doses that were observed in these studies.

Other dopamine agonists which were found to cause consistent increases in the activity of pallidal neurons are lisuride and pergolide. While many of these effects of the dopamine agonists on pallidal activity may be indirect, mediated through interactions of the drugs with dopamine receptors in the striatum on neurons projecting to the external pallidus, we observed that dopamine iontophoresed onto cells in the globus pallidus, as in the substantia nigra pars reticulata, partially blocked the ability of GABA to inhibit pallidal activity. Since there is large GABAergic innervation of the globus pallidus cells, originating in the striatum, this observation suggests that the dopamine agonists may also increase pallidal activity by attenuating the effects of GABA on these neurons. Neither norepinephrine nor acetylcholine had similar effects on GABA's ability to inhibit pallidal activity indicating that this effect is, at least to some extent, specific for dopamine.

These observations support our findings in the substantia nigra pars reticulata and suggest that dopamine agonists may act, in part, by stimulating these dopamine receptors which appear to be mediating a modulation of GABA's response. To date we have found that systemic administration of apomorphine does cause a partial block of GABA's ability to inhibit pallidal activity when this inhibitory neurotransmitter is iontophoresed onto pallidal neurons. This ability of dopamine to modulate the effects of other neurotransmitters may partially explain the confusion which has arisen over whether dopamine is an excitatory or inhibitory neurotransmitter. Like norepinephrine and serotonin, its action at some sites may depend on what other neurotransmitters are being released at the same place and time.

### (c) Substantia Nigra Pars Compacta Studies

In previous years we have examined the effects of different types of dopamine agonists on the activity of dopamine cells in the pars compacta of the substantia

nigra. The dopamine neurons have proved to be very sensitive to the systemic effects of the more classic dopamine agonists like apomorphine, and the newer ergot derivatives, such as lisuride and lergotriole. For the most part, the inhibitory effects of these agonists on the dopamine neurons appear to be mediated directly at the level of dopamine autoreceptors, since destruction of the striato-nigral feedback loop has little (lisuride) or no significant (apomorphine) effect on the ability of these agonists to inhibit dopamine cells.

In the past year, we have begun to examine the effects of some of the newer, more selective dopamine agonists. 3-(3-Hydroxyphenyl)-N-n-propylpiperidine (3-PPP), a putatively selective presynaptic dopamine receptor agonist, was found to inhibit dopamine cell activity effectively when given systemically with a potency 12 times less than that of apomorphine. This agonist seems to have little effect on pallidal activity. These observations support the claims that this agonist is more effective at presynaptic dopamine receptors than at postsynaptic dopamine receptors.

The Section has also been conducting an investigation of the effects of sub-chronic administration of L-dopa on the ability of dopamine neurons to respond to apomorphine, amphetamine and L-dopa. The L-dopa has been administered twice a day (250 mg/kg) with a decarboxylase inhibitor, for either 5 or 12 days. These studies were designed to gain some insight into the effects of chronic dopamine agonist administration on the sensitivity of dopamine autoreceptors and on the dynamics of dopamine synthesis and turnover. They were also designed to explore the possibility, suggested in the literature, that chronic L-dopa treatment causes dopamine receptors to become supersensitive. We have found that the dopamine cells show decreased responses to a challenge dose of amphetamine and L-dopa after both 5 and 12 days of L-dopa pretreatment and that they also show a decreased response to apomorphine after 12 days of L-dopa. These studies suggest that the dopamine receptors are becoming subsensitive, rather than supersensitive, with chronic L-dopa treatment. However, they suggest, in addition, that some processes which mediate the effects of amphetamine and L-dopa, such as dopamine storage and release, and perhaps dopamine synthesis as well, are also being affected by the chronic agonist treatment. The biochemical aspects of these studies are being performed in collaboration with Dr. Leonard Miller (NHLBI).

## (2) Physiological effects of GABA and GABA agonists in the basal ganglia.

We have previously shown that the cells in the substantia nigra pars reticulata are sensitive to the inhibitory effects of both i.v. and iontophoretically applied muscimol and THIP, potent GABA agonists, and to iontophoretically applied GABA. In the past year, we have demonstrated that cells in the globus pallidus, which also receives a substantial GABAergic projection from the striatum, show similar sensitivities to these agents.

Since it has been postulated that the central effects of the benzodiazepines are mediated by the ability of the benzodiazepines to potentiate the inhibitory effects of GABA on neuronal activity, we have examined the actions of diazepam and flurazepam on the activity of substantia nigra pars reticulata cells. When administered systemically, both drugs exert inhibitory effects on the activity of these neurons; flurazepam is considerably less potent than diazepam. This



difference in potency may reflect differences in lipid solubility and kinetics of distribution. They are consistent with observed differences in clinical efficacy. For each drug, the variability in the degree of inhibition may be related to differences in tonic GABAergic input among neurons, to different levels of an endogenous ligand for the benzodiazepine receptors. The inhibitory effects of these two benzodiazepines on reticulata cells are of the same order of magnitude as those on locus coeruleus neurons. The benzodiazepines may thus have multiple clinically relevant sites of action. The benzodiazepine studies have been conducted in collaboration with Dr. Richard Ross (NIGMS-NIMH).

#### Proposed Course:

(1) Physiological effects of dopamine and dopamine agonists in the basal ganglia.

##### (a) Substantia Nigra Pars Reticulata Studies

In the coming year we plan to investigate further the significance of the ability of dopamine to modulate the actions of other neurotransmitters. We will specifically investigate the question of whether dopamine can modulate the effects of excitatory neurotransmitters, such as acetylcholine and glutamine, on the cells of the pars reticulata. We also plan to investigate whether this action of dopamine is physiological. Can amphetamine, administered systemically or injected directly into the substantia nigra, cause a change in the efficacy of iontophoresed GABA (or glutamate) by releasing dopamine from dopamine dendrites in this region? Such an effect would support the idea that the dopamine dendrites have the capacity to release dopamine and activate this modulatory mechanism. We will also investigate the pharmacological implications of this action of dopamine. Does systemic administration of dopamine agonists and antagonists bring about a change in the efficacy of other neurotransmitters on the reticulata neurons? In addition, we will explore the indirect, striatally mediated, effects of dopamine and dopamine agonists on these cells. We will try to learn more about the nature of the dopamine receptors mediating these various effects by examining the actions of dopamine agonists and antagonists thought to be selective for the different subcategories of the dopamine receptor.

##### (b) Globus Pallidus Studies

To explore the ramification and general significance of dopamine's apparent ability to modulate the actions of other neurotransmitters in the CNS, we will carry out experiments in the globus pallidus like those described above for the substantia nigra pars reticulata. Taken together with the results observed in the substantia nigra and other brain regions, these studies may lead to an alteration in our concept of how dopamine affects information processing in the basal ganglia.

In the globus pallidus we will also investigate further the interesting observation that the schedule of administration influences the magnitude of response to a dopamine agonist by determining whether similar phenomena are observed with dopamine agonists other than apomorphine. We will examine the effects of both agonists and antagonists which are specific for different categories of dopamine receptors to attempt to sort out which types of receptors are mediating the responses we see and to gain insight into how neuroleptics affect the activity of the pallidal neurons. We will try to determine where dopamine drugs act to affect

pallidal activity through a combination of iontophoretic, lesion and local injection techniques. In addition, we are interested in determining how responses of the pallidal cells to dopaminergic drugs may be effected by chronic dopamine agonist treatment and by lesion of the dopamine pathway with 6-hydroxydopamine.

### (c) Substantia Nigra Pars Compacta Studies

We will continue to explore the effects of new dopamine agonists with potential for stimulating specific subcategories of dopamine receptors on dopamine cells in the pars compacta of the substantia nigra. We plan to set up a pressure iontophoresis system in order to better investigate the effects of these drugs when they are directly iontophoreted onto the dopamine neurons. Our current iontophoretic techniques do not work very effectively in the dopamine system and with many of the ergot agonists. We will continue to attempt to correlate the biochemical effects of chronic L-dopa treatment with the effects of this treatment on the single unit activity of dopamine neurons.

### (2) Physiological effects of GABA and GABA agonists in the basal ganglia.

We will explore further the interactions between the benzodiazepine drugs and the GABA system in the pars reticulata of the substantia nigra. We will investigate the effects of purines in this system in view of the possibility that some purines may serve as endogenous antibenzodiazepine agents. We also hope to explore the actions of an antibenzodiazepine compound recently described by Hoffman La Roche, to gain insight into the significance of the role of the endogenous benzodiazepines in this region.

### Publications:

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ANNUAL REPORT

October 1, 1980 through September 30, 1981

Neuroimmunology Branch

National Institute of Neurological and Communicative Disorders and Stroke

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Annual Report  
October 1, 1980 to September 30, 1981  
Neuroimmunology Branch  
National Institute of Neurological and  
Communicative Disorders and Stroke

Dale E. McFarlin, M.D., Chief

Research in the Neuroimmunology Branch (NIB) is directed at the assessment of immune mechanisms operative in neurological diseases. These investigations include both studies of experimental neurological diseases in animals and studies of patients with neurological diseases with a possible immunological basis. Particular emphasis is placed upon the use of pure and well characterized antigens and the study of genetic factors which control the immune response.

The investigations of monozygotic and dizygotic twins who are either concordant or discordant for multiple sclerosis have been expanded. In the past both clinical and spinal fluid findings in discordant twins have suggested that this population may have a high prevalence of subclinical disease. Over the past year, longitudinal studies tend to support this suspicion. Three individuals previously classified as normal or as possible multiple sclerosis have developed definite disease.

Cellular immune function is being extensively studied in patients with multiple sclerosis and specifically in the twins. The cellular response to measles, mumps and vaccinia has been evaluated using a lymphoproliferative technique. Although a substantial response can be demonstrated to mumps and vaccinia virus, only marginal proliferation can be demonstrated to measles virus in most individuals despite the presence of serum antibody to this virus. Although the explanation for the low cellular response to measles has not been established, six individuals who clearly respond to measles virus have been identified. It is noteworthy that five of the six individuals have multiple sclerosis. The individuals who respond to measles virus in a lymphoproliferative assay also develop cytotoxic killer cells directed at the virus which indicates that the lack of responsiveness to measles virus is not limited to one particular technique. However, different subpopulations of thymus-derived lymphocytes may be involved in each assay because the proliferative response does not require that the responding lymphocytes and the measles bearing targets share histocompatibility antigens. In contrast, in the measurement of cytotoxic cells HLA compatibility is necessary.

Three of the individuals who respond to measles virus are members of twin sets in which the nonresponder twin does not have multiple sclerosis. These HLA identical responder and nonresponder twin sets establish that the lack of a response to measles is not due to the genetic background and provide an opportunity to seek other mechanisms. Mixing experiments have been performed seeking suppressor mechanisms as an explanation for the lack of a response in the nonresponder individual. To date, no evidence of this has been found.

The subset of cells which react in the responding individuals has been identified as the T gamma cell fraction. In mixing studies from responder and nonresponder twins it has been demonstrated that the nonresponder lacks one of two cell populations necessary for proliferation against the virus. These data indicate that there is a failure in the sensitization or maintenance of immunological memory to measles virus in one T cell population in most individuals. In addition, the findings suggest that this subpopulation persists in the patients with multiple sclerosis who respond to the virus. This may be due to differences in the biology of the infection in these patients or to underlying defects in the immuno-regulatory function that have not been elucidated.

In addition to assessing the immune response to viruses our research includes the study of immunoglobulin synthesis in vitro, the production of interferon by lymphocytes, and the effect of interferon upon lymphocytes. In all of these investigations when differences between normal individuals and patients with multiple sclerosis are encountered attempts are made to elucidate the mechanism by mixing experiments similar to those described above.

Work on experimental allergic encephalomyelitis (EAE) has continued and of particular importance has been the development of a new method which enables the reproducible production of this disease in mice. Many fundamental principles of basic immunology have been established in the mouse and having a reproducible experimental autoimmune disease in this species should enable the characterization of the immunological events resulting in CNS disease. It is of considerable interest and possible relevance to human diseases that many of the mice develop a chronic relapsing and recurrent form of EAE. Initial pathological studies have shown that such animals have central nervous system lesions of different ages, chronic and acute demyelination as well as other features in common with human demyelinating diseases such as multiple sclerosis.

Studies on the immune response to measles virus in patients and in mice with persistent CNS infections due to this virus have been continued. Emphasis is being placed on the surface antigens coded for by the virus which are also expressed on infected cells. This aspect of our work has been greatly facilitated by the successful production of a number of monoclonal antibodies against the principle surface glycoprotein, the measles hemagglutinin. Studies with these antibodies have resulted in a number of significant observations: 1) Although the various monoclonal antibodies vary in immunochemical and biological activity, in general they neutralize virus infectivity and block hemagglutination which provides formal proof that these activities are related to the HA protein. 2) By using purified monoclonal anti-HA protein it has been possible to purify this important antigen. The pure virus protein retains hemagglutinating activity and the capacity to react with antibody and in certain humans, discussed above, it evokes a cell-mediated immune response. 3) Studies in progress are directed at the detailed examination of synthesis, transport and insertion of this antigen in

infected cells. 4) The distribution of HA-antigens among various strains of measles virus is being addressed. The preliminary data indicate that in comparison to the hemagglutinin antigen of the influenza virus, there appears less variation in the measles virus hemagglutinin. However, in the past it has been believed that lack of variation in the measles HA allows the production of lifelong immunity to this virus. In fact, even our data indicate that this is not the case and some determinants recognized by individual monoclonal antibodies are clearly undetectable in certain strains of measles virus. In addition, no cross-reactivity between canine distemper virus and bovine rinderpest virus has been demonstrated, although these paramyxoviruses are known to be closely related. 5) One of the monoclonal antibodies modifies acute measles encephalopathy and leads to persistent virus infections in mice. Our work in the past has shown that mice infected with HNT strain of measles virus die acutely. If three days after infection the mice are given hyperimmune anti-measles antibody the animals do not die and a significant percentage get chronic neurological disease. Thus, an obvious question, was which component of the anti measles antisera was responsible? One of the monoclonal antibodies to HA protein produces the same effect. Furthermore, serial studies of antigen in the brain have shown that treatment with the monoclonal anti-HA antibody leads to reduced viral synthesis by the infected host. Thus, reactivity to a surface component of virus infected cells modifies the intracellular molecular events. This observation has direct implication on the use of antibody to treat acute infections of man, and in fact, we have recently investigated a patient who was given hyperimmune antibody against measles while incubating the virus and subsequently developed subacute sclerosing panencephalitis.

The above work with monoclonal antibody to the HA protein of measles virus in addition to providing new information demonstrates that these approaches are powerful tools for investigation of trace substances in normal and infected cells of the nervous system. The insight gained from these studies obviously has direct application to the investigation of other important areas in the neurosciences.





Project Description:

Objective:

A. Studies of Multiple Sclerosis (MS)

The goal of this project is to establish a more precise understanding of genetic and immunological factors in multiple sclerosis. These factors are being studied in clinically well-defined cases of MS. Numerous interrelated questions are being investigated and include:

1. Examination of the histocompatibility make-up in a population of sporadic cases of MS and appropriate control individuals.

2. Identification of lymphocyte antigens occurring with increased frequency in MS and correlation of these antigens with established genetic and immunological parameters.

3. Evaluation of the cellular immune response to a number of antigens including viruses, components of the nervous system and histocompatibility antigens. This also includes investigation of lymphocyte populations and subpopulations responding to these various antigens.

4. Correlation of histocompatibility make-up with the cellular and humoral immune response to various viral antigens.

5. Evaluation of cerebrospinal fluid (CSF) immunoglobulin content.

6. Determination of lymphocyte phenotypes during different phases of the disease.

7. The above investigations are being carried out in families with a multiple incidence of MS and in monozygotic and dizygotic twins concordant or discordant for MS. The segregation of histocompatibility antigens and differences in immune function between affected and nonaffected individuals are being studied in both families and twins.

B. Studies of Subacute Sclerosing Panencephalitis (SSPE)

Because of the established relationship of SSPE to measles, humoral and cell-mediated immunity (CMI) in this disease are being assessed in a few patients.

Methods Employed:

Patient Populations. All patients included in these studies have been evaluated as either inpatients or outpatients on the NIB service at NIH. Each individual receives a complete medical and neurological evaluation with appropriate diagnostic studies. Patients classified as possible for definite MS are included in the studies.



Studies of familial MS involve families with two or more clinically confirmed cases of MS. Twins either concordant or discordant for MS are being admitted to the Clinical Center in order to document the clinical aspects of each case, to perform extensive laboratory evaluation, and to clinically classify each pair. These studies are performed in collaboration with Roswell Eldridge, M.D., Clinical Neurogenetics Studies, IRP. SSPE patients who have the characteristic clinical EEG, CSF and serological findings are studied.

Histocompatibility. Histocompatibility testing for HLA-A and HLA-B antigens are being performed under contract by Dr. Paul Terasaki. HLA-D typing for DW2 antigens is done by mixed lymphocyte culture in our laboratory. Sera from multiparous wives or mothers of patients are being employed in cytotoxicity assays to identify antigens on T or B lymphocytes from MS patients.

Humoral Immunity. Conventional assays (CF, HAI and neutralization) for antibody to various viruses including measles, rubella, mumps, and vaccinia are performed on serum and CSF. Antibody to these viruses is also measured in serum and CSF using radioimmunoassay.

Cell-Mediated Immunity. Cell-mediated immunity to viral antigens are studied in patients and controls using a lymphocyte stimulation assay. The CMI response to measles virus is also studied using purified viral antigens and macrophage inhibition assays. The responses of T lymphocytes and B lymphocytes are studied in these assays. These populations are prepared from peripheral blood using immunoabsorbant columns and rosetting methods. T-cell subpopulations are obtained on the basis of their capacity to react with Fc receptors of IgG and are described more fully in project Z01 NS 02205-06 NI.

Cerebrospinal Fluid. IgG, IgM and IgA levels are quantitated in each CSF by radioimmunoassay. CSF is also being examined for the presence of oligoclonal IgG in collaboration with Infectious Diseases Branch.

Surface Markers. Lymphocyte phenotypes are determined with monoclonal antibody directed against determinates on the surface of lymphocytes which correlate with the functional activity of cell populations in certain in vitro system. The lymphocytes bear each surface antigen are quantitated by analysis with a FACS IV. Commercially available antisera are used for these studies. In addition, efforts are underway to produce monoclonal antisera which define additional lymphocyte subsets.

#### Major Findings:

1. A major emphasis of the clinical research program has been an extensive study of monozygotic and dizygotic twins who are either concordant or discordant for MS. This represents an extension of investigations of familial MS and was undertaken to identify more precisely the importance of

genetic and environmental factors in the disease. Thirty sets of twins have been evaluated. The degree of concordance was six of twelve in the monozygotic as opposed to two of twelve in the dizygotic pairs. This observation that concordance is higher in monozygotic twins supports the concept that a genetic factor may play a role in the pathogenesis of the disease. However, the absence of complete concordance in the monozygotic twins emphasizes the importance of other factors.

Our initial evaluations showed soft clinical findings and CSF abnormalities in many of the "normal" twins. This suggested a high incidence of subclinical disease in this population. During longitudinal studies over the past year, three individuals originally classified as normal or possible multiple sclerosis have developed definite disease. This adds to support to the suspicion of a high incidence of subclinical disease.

2. The cellular immune response to viruses is being assessed in the twins. During the initial evaluation, considerable difference in the immune response to certain viruses was found in some twins having the identical genetic background. A small number of individuals who give significant response to measles virus have been identified and two responding individuals are members of twin sets in which the other twin member fails to respond to measles virus. These responder and nonresponder twin sets have provided an opportunity to perform mixing experiments to identify suppressor mechanisms in the nonresponder individual. No evidence of suppression could be identified. In the responding individuals, the reactive cells are limited to the T<sub>H</sub> cell subpopulation. In the twin mixing studies it was demonstrated that the nonresponder lacks one of two cell populations necessary for proliferation to measles virus. These studies suggest that there is a failure in sensitization or maintenance of memory to measles virus in one T-cell population in most individuals. A small number of patients and one control appear to maintain this measles virus specific T-cell subpopulation. This may be due to differences in the biology of infection in these individuals. The presence of responder and nonresponder identical twin sets eliminates a genetic basis for this difference.

3. Recent reports have indicated that multiple sclerosis patients have a low proportion of lymphocytes bearing the OKT8 marker as compared to those bearing the OKT4 marker. This observation is generally consistent with studies which indicate an abnormality of suppressor cell activity in multiple sclerosis patients. Such studies are being performed on the patients which have been followed in the Neuroimmunology Branch and their appropriate controls. Our findings to date are somewhat preliminary but generally consistent with those of other laboratories. These investigations have also been carried out in the twin population and based on the concept that multiple sclerosis patients may lack T suppressor activity, a preliminary treatment protocol has been initiated. In this treatment plan lymphocytes are removed from the monozygotic unaffected twin by leukopheresis and infused in the affected twin. This procedure has recently been carried out in one twin set and the results are currently being evaluated.

4. Findings in SSPE. As described in Project Z01 NS 02205-06 NI, the administration of anti-measles antisera to mice inoculated with measles abolishes the acute disease, but many animals develop chronic encephalitis. A patient has recently been studied by us, who was given hyperimmune anti-measles globulin while incubating the virus. This individual had a mild clinical measles infection but subsequently developed SSPE. This sequence of events suggest that the antibody may have modified the acute disease and played a role in the development of chronic infection.

The antibody specificities of the SSPE patients did not differ from that seen in serum from normal individuals after acute measles infection. Of particular interest was that reactivity against the matrix protein was found. In some patients with SSPE the amount of antibody against the matrix protein was relatively reduced. However, this was also true of the serum from some normal individuals convalescing from acute measles infection and from patients with chronic active hepatitis.

#### Significance to Biomedical Research and the Program of the Institute:

The total effort in this project is directed toward the investigation of human diseases of the nervous system. Contributions from other basic projects within the Branch are applied to the study of clinical problems. The majority of the effort is aimed at understanding mechanisms involved in multiple sclerosis. This is a major health problem which affects young individuals at the prime of life. Over the past years a number of fragmentary bits of evidence, suggesting possible etiologies and factors contributing to pathogenesis have been put forth. The present investigation is aimed at intensive study of a small group of well-characterized patients. In addition, the use of families in which there is more than one case of multiple sclerosis and twins eliminate some of the variables encountered in studying sporadic cases. Although SSPE is a rare disorder, it is important because of the documented association between this disease and measles virus. Defining the parameters of the immune response to measles may not only provide insight to the pathogenesis of SSPE, but in addition this may provide important information about other disorders of unknown etiology.

#### Proposed Course:

Work on this project will be directed at establishing a more precise understanding of the findings previously described. This work will focus on characterization of lymphocyte subpopulations in patients with multiple sclerosis. Studies of human lymphocyte subsets with monoclonal antibodies will be expanded. These will be used to seek differences in lymphocyte subpopulations in patients with acute chronic forms of multiple sclerosis. This approach will be used to analyze lymphocyte subpopulations in the twins, particularly those who show differences in

the immune response to viral antigens. The population of twins which has been evaluated will be followed for the development of new symptomatology. In addition efforts will be made to study lymphocytesubpopulation in normal individuals and disease control. Additional sets of twins will be added to the series. Particular emphasis will be placed on obtaining monozygotic discordant twins beyond the age of 50. The therapeutic approach which involves removal of lymphocytes from a normal individual and infusing these into an identical twin sibling with multiple sclerosis will be expanded.

Publications:

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02203-06 NI
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less) The Immune Response Against Membrane Antigens		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:   OTHER:	D.E. McFarlin J.F. Poduslo W.J. Bellini H.F. McFarland J.M. Gheuens C.L. Koski A. Trudgett	Chief Staff Fellow Staff Fellow Asst. Chief Visiting Assoc. Guest Worker Visiting Assoc.
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COOPERATING UNITS (if any)		
LAB/BRANCH Neuroimmunology		
SECTION Neurological Diseases Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD 20205		
TOTAL MANYEARS: 3.0	PROFESSIONAL: 2.5	OTHER: 0.5
CHECK APPROPRIATE BOX(ES) <input type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input checked="" type="checkbox"/> (c) NEITHER <input type="checkbox"/> (a1) MINDS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords)  The goal of this project is to characterize the immune response to components of <u>myelin</u> and of measles virus. This study has focused on the composition of the <u>outer membrane surface</u> . <u>Glycoproteins</u> have been identified in the <u>myelin complex</u> by the binding of lectin to components electrophoretically separated. Monoclonal antibodies to a major surface component of measles virus, the hemagglutinin, have been produced and used to purify this glycoprotein.		



Project Description:Objectives:

Measles virus encodes for two polypeptides, the hemagglutinin and fusion proteins, which are expressed on the surface of the virus and are inserted into the plasma membrane of infected cells. The immune response to these antigens may play a role in susceptibility or resistance to disease. A major objective of this study is to assess the immune response to these components with respect to both humoral and cell-mediated reactivity. Animals with experimental measles infection or hyperimmunized with inactivated virus are being used to develop methods applicable to the study of normal individuals as well as patients with chronic disease such as subacute sclerosing panencephalitis (SSPE) and multiple sclerosis (MS). Another objective is to prepare monoclonal antibodies against these antigens, and to use these antibodies to characterize these viral antigen components both biologically and chemically.

The surface components of the myelin sheath and the oligodendroglial plasma membrane are important in attempting to define alterations which occur in in demyelinating disorders, such as multiple sclerosis. Such components may not only play important recognition roles in the process of myelination or myelin maintenance, but in addition may be readily susceptible to immunological damage or even act as specific viral receptors. Consequently, the identification, isolation and immunological characterization of these surface membrane components is another aim of this research.

Methods Employed:

## A. Measles virus membrane antigens

Plaque-purified Edmonston measles virus is grown in Vero cells to titers exceeding  $1 \times 10^7$  PFU/ml. Supernatant virus is first concentrated by ultrafiltration and purified by two successive velocity sedimentations in sucrose. During virus replication in Vero cells, the various components of the virus may be specifically radiolabeled with nucleotides, sugars and/or amino acids depending upon the intent of the study.

Two cell lines, MA160 and HEp2, persistently infected with the mantooth and Edmonston strains, respectively, are also being used. In addition, the Hamster Neurotrophic (HNT) strain is also being studied. In various experiments virus components are internally labeled with either radioactive amino acids or carbohydrate. The outer membrane components are radioiodinated with lactoperoxidase.

Mice hyperimmunized with measles virus are being used to prepare monoclonal antibodies. Two days after an intravenous boost, spleens are removed and a cell suspension prepared. Cells are fused with 8-azaguanine resistant murine myeloma cell lines using polyethylene glycol. The resulting hybrids are screened for antibody formation by radioimmunoassay. Those which produce antibody are cloned by limiting dilution or in soft agar. Positive clones are grown in tissue culture to yield quantities of antibody sufficient for our studies. In addition, antibody-producing cells are injected into the peritoneal cavity of syngeneic mice in order to obtain ascites fluid rich in antibody.

In order to purify the virus hemagglutinin monoclonal antibody against this protein is precipitated with  $(\text{NH}_4)_2\text{SO}_4$ . The immunoglobulin is dialyzed and coupled to Sepharose with cyanogen bromide. These anti HA-Sepharose beads are used to purify the HA protein. Membrane fraction from cells infected with measles are enriched by floatation gradients and passed over the anti HA-Sepharose beads which are subsequently washed extensively and eluted with caotrophic agents.

The purified HA protein is analyzed by slab gel electrophoresis and used for immunological and chemical studies. The latter included experiments designed to identify the number and location of antigenic sites in the molecule. In these studies the purified antigen is digested by proteolytic enzymes and the resulting fragments analyzed by various monoclonal antibodies. The immunological studies consist of elucidating the capacity of purified HA protein and its various fragments to react with antibody and lymphocytes from animals immunized with virus or patients with neurological diseases.

The antibody to measles virus is measured by solid phase RIA. The response to the individual polypeptides of the virus is determined by a solid phase radioimmunoassay performed in polyacrylamide gels and by immunoprecipitation of internally labeled viral polypeptides followed by fluorographic assessment subsequent to polyacrylamide gel electrophoresis and subsequent fluorography.

The biological activity of various monoclonal antibodies is determined by neuralization and hemagglutination inhibition assays. In addition radioimmunoassay and immunofluorescence are being used to seek reactivity between monoclonal antibody and a number of measles strains as well as canine distemper and rinderpest.

#### B. Membrane myelin

The brains from adult Lewis rats are rapidly removed and used immediately for the purification of myelin according to the standard procedure. Aliquots of the isolated myelin are solubilized at a concentration of approximately 1 mg protein/ml of sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis buffer (w/v) bromophenol blue, and 100/o (v/v) glycerol.

In some experiments, the isolated myelin is extracted with chloroform/methanol (2/1, v/v) to concentrate the high molecular weight proteins. This was accomplished by successive extractions (3x) of myelin at a concentration of approximately 0.5 mg of myelin proteins per ml solvent. After each extraction the remaining myelin proteins are concentrated by centrifugation.

A programmable gradient maker is used to prepare linear gradients of polyacrylamide gel in a vertical slab gel system. Samples of solubilized myelin containing approximately 30  $\mu$ g protein are applied to an 8 x 25 mm well in sample volumes of 10-100  $\mu$ l. Electrophoresis is carried out for 4 hours. The slab gels are fixed in methanol/water/acetic acid (45:45:10, v/v/v) and either used for the lectin binding studies or stained in 0.20% (w/v) Coomassie Blue dissolved in this fixative. Stained gels were destained overnight, dried and photographed both before and after drying. Proteins of known molecular weights are radioiodinated by the chloramine T procedure and used as standards. Appropriate aliquots were electrophoresed to provide adequate detection by autoradiography.

Three lectins, wheat germ agglutinin, concanavalin A, and soybean agglutinin, were iodinated under similar conditions in the presence of their respective inhibitory monosaccharides, (N acetyl-D-glucosamine, N acetyl-galactosamine and D-mannose, D-glucose, respectively). Specific activities of the iodinated lectins were determined. Lectin binding to individual proteins is assessed by direct application of the [ $^{125}$ I] lectins to the gradient slab gel after electrophoresis. The gels are sliced longitudinally into lanes which contained the separated proteins used for the lectin binding experiments. The gel slices are placed in humidified plastic dishes and overlaid with the iodinated lectins diluted in the appropriate buffer. After incubating for 18-24 hrs, the slices are washed in buffer, dried and analyzed by autoradiography by varying the exposure times between 4 hrs. and 2 weeks to optimally visualize the bands. In some experiments, non-specific binding is controlled for by incubating and washing the gels in the presence of the inhibitory monosaccharide. Photographs of the gels and autoradiographs are enlarged to 8 x 10 inches, and the relative mobility of each protein measured directly by determining the ratio of the distance migrated by a given protein and the gel length.

#### Major Findings:

##### A. Measles Membrane Antigens

A purification procedure for measles virus has been developed which maintains the infectious nature of the virus as well as the antigenicity of the viral polypeptide components. Between 2 and 3 mg of virus is routinely recovered from 3 to 4 liters of supernatant fluids.

Fluorographic studies using either  $^{35}\text{S}$  methionine or  $^3\text{H}$  glucosamine labeled virus indicate that purified virus is composed of 6 major structural polypeptides. To date, only a single glycoprotein has been identified, ie, the HA or hemagglutinin. This has been confirmed using specific lectins in the gel overlay method. The second surface protein, the fusion protein has a molecular weight of reduced SDS gels of 41,000 daltons and presumably is associated under non-reduced conditions with an 18,000 dalton glycopeptide. The core polypeptides include the nucleocapsid, the phosphorylated nucleocapsid associated "P" protein as well as the M or matrix and L or presumptive polymerase polypeptides.

Monospecific antibody to the measles polypeptides has been obtained by hybridoma technology. Thus far, three hybrids that produce antibody to the hemagglutinin of measles have been cloned. One of these, the C1 clone, has been rigorously characterized. The antibody reacts exclusively with the HA polypeptide in immune precipitation, inhibits hemagglutination, neutralizes measles infectivity and is IgG<sub>1</sub>K. The antibody has been purified from ascites fluids and has been coupled to sepharose with cyanogen bromide. This solid phase immune absorbant has been used to purify the HA protein. Afterwards it retains biological and immunological properties. For example, the purified antigen agglutinates monkey red cells. On a theoretical bases such activity would require a polymeric state. The results of velocity sedimentation studies support the presence of such a form. It seems likely that the hydrophobic portions of purified HA bind to each other. The pure HA protein reacts with antibodies to measles and stimulates lymphocytes from responder individuals to proliferate. These findings document that both the humoral and cellular components of the immune response are directed at determinants on the HA protein.

The C1 monoclonal anti-HA antibody reacts with some but not all strains of measles virus; it does not react with three strains of canine distemper virus and rinderpest virus which are closely related paramyxovirus.

#### B. Myelin Membrane

In order to analyze the molecular composition of myelin it was essential to develop new methodology which would provide reliable molecular weights of individual components over a wide range and secondly to detect glycosolated components which were present in trace amounts. Pore gradient electrophoresis in the presence of SDS produced high resolution of multiple proteins and permits estimation of molecular weights ranging from  $10^3$  to  $10^6$  daltons. Several methods were used to estimate molecular weights. A linear relationship between the logarithm of the molecular weight [ $\log(\text{MW})$ ] and the logarithm of the relative mobility [ $\log(\text{RM})$ ] was found; however, this relationship only remained linear over 30-fold range of molecular weights. Linearity over



a wider molecular weight range was found in the relationship between  $\log(MW)$  and logarithm of the gel concentration at the position reached by the macromolecule  $\log(O/T)$ . A computer program was developed which provides statistical estimation of the molecular weight for an unknown protein together with the standard error and 95% confidence. In MW weight ranges in which the  $\log(MW)$  and  $\log(RM)$  were non-linear least-square-curve-fitting provided satisfactory estimates of molecular weights.

In order to analyze the glycosylated components of myelin, purified myelin was separated by gradient slab gel electrophoresis. Subsequently radioiodinated lectins were applied and the glycoproteins identified by autoradiography. Using iodinated wheat germ agglutinin, soybean agglutinin and concanavalin A, a total of 22 lectin binding components were identified.

#### Significance to Biomedical Research and the Program of the Institute:

Myelin is an important component of the nervous system. Characterization of the molecular components should provide insight into the function of the membrane under normal conditions. Elucidation of surface antigens should provide clues of possible abnormalities in diseases involving peripheral and central myelin. Measles virus is a ubiquitous infectious agent of man, producing in most cases a self-limiting disease. Serious complications can arise including pneumonia and encephalitis. This paramyxovirus appears capable of establishing a persistent infection which leads to a slowly progressive disease, SSPE and has been implicated in multiple sclerosis. In addition, widespread immunization with live virus is currently practiced nationwide. Little is known about those components of measles virus which invoke a humoral and/or cellular immune response in man. Through examination and comparison of the immune response elicited in normal individuals with that of individuals suffering from measles-induced disease states, certain differences may appear which are important in understanding the mechanisms leading to disease. Emphasis has been placed on the purification and chemical characterization of the membrane antigens due to the accessibility of these components to the immune system and the feasibility for these proteins to alter normal surface topography.

#### Proposed Course:

Efforts will be made to prepare additional monoclonal antibodies directed at other surface components of both myelin and measles. These will enable investigation of the cellular and humoral immune response to measles virus, as well as the components of the myelin membrane, in experimental and human diseases. Continued efforts will be made in the purification and chemical characterization of the individual membrane



components by conventional means as well as hybridoma immunoabsorbents. Such efforts are paramount to the understanding of the interaction between the immune recognition systems and membrane surfaces. The intracellular synthesis of measles polypeptides in lytically and persistently infected cells will be characterized.

Publications:

Bellini, W.J., McFarlin, D.E., Silver, G.D., Mingioli, E.S. and McFarland, H.F.: The hemagglutinin of measles virus. I. Immune reactivity of the purified hemagglutinin of measles virus. Infection and Immunity 32: 1051-1057, 1981.

Trudgett, A., Gould, E.A., Armstrong, M., Mingioli, E.S. and McFarlin, D.E.: Antigenic differences in the hemagglutinin of measles and related viruses. Virology 109: 180-186, 1981.

Yahara, S., Kishimoto, Y. and Poduslo, J.: High performance liquid chromatography of membrane glycolipids. In Sweeley, C.C. (Ed.): Cell Surface Glycolipids, District of Columbia, American Chemical Society, 1980, pp. 15-33.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02204-06 NI
PERIOD COVERED October 1, 1980 to September 30, 1981		
TITLE OF PROJECT (80 characters or less) Immunologic Mechanisms Operative in Experimental Autoimmune Diseases of the Nervous System		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:  OTHER:	D.E. McFarlin C.B. Pettinelli A.M. Brown R.H. Schwartz R. Fritz	Chief Sr. Staff Fellow Guest Worker Sr. Investigator IPA     NI NINCDS NI NINCDS NI NINCDS LI NIAID NI NINCDS
COOPERATING UNITS (if any) Departments of Pathology (Neuropathology) and Neuroscience, Albert Einstein College of Medicine, New York, NY LI, NIAID		
LAB/BRANCH Neuroimmunology		
SECTION Neurological Diseases Section		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, MD 20205		
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SUMMARY OF WORK (200 words or less - underline keywords)  The aim of this project is to identify the relative role of various mechanisms operative in the production of experimental allergic <u>encephalomyelitis</u> , a model of autoimmune disease. The immune response to <u>myelin basic protein</u> is being assessed by measuring antibody and in vitro proliferative responses in various strains of mice. The relationship of the genetic background to disease expression is being extensively evaluated. The antigenic components responsible for disease production, T-cell proliferation, and reactivity with antibody are being studied. Chronic recurrent disease has been produced in mice. The pathology in affected animals is being characterized.		

Project Description:

**Objective:** EAE, a model autoimmune disease of the CNS, has a number of clinical, histological, and immunological features similar to human demyelinating diseases and to chronic encephalitis. The object of this project is to delineate the mechanisms responsible for the pathogenesis of EAE. Susceptibility to EAE and the immune response to myelin basic protein (BP), the established encephalitogenic antigen in a number of species, is being studied in various strains of mice with different genetic backgrounds in order to elucidate the relationship between histocompatibility background and disease mechanisms. Two types of disease are being studied in mice. One is an acute form which is produced by the adoptive transfer of sensitized lymphocytes. The second, which has recently been developed in our laboratory, has a chronic relapsing course.

Methods Employed:

These investigations are being performed in the mouse because this species is well characterized immunologically and because other research in the NIB is being conducted in this species (Project Z01 NS 02205-05 NI). A number of inbred strains of mice are challenged with either whole central nervous tissue or BP in various adjuvants. BP from several sources is being employed. Clinical disease, CNS pathology, cell-mediated immunity and antibody formation are evaluated.

Lymphocyte stimulation (LS), macrophage migration inhibition and cytotoxicity are being used to assess cellular immune function *in vitro*. Optimal culture conditions have been established for the assays which are performed in conjunction with column separations and transfer experiments described below. Both lymph node cells (LNC), and spleen cells (SC) are used. Anti-BP antibody is measured by solid phase RIA.

Lymphocytes are separated into T-cell and B-cells with nylon wool columns and immunoabsorbant column with anti-IgG coupled to Sephadex. Lymphoid cells are treated with monospecific antisera and complement to deplete certain subpopulations of T-cells and to enrich for others. Lymphoid cells, separated by these methods, are characterized by FA, cytotoxicity, and LS as measured by thymidine incorporation.

LNC, SC and purified subpopulation of cells are evaluated for the ability to transfer EAE into normal syngeneic animals. The encephalitogenic responses versus dose of transferred cells is determined by clinical and histological grading of the recipients.

Major Findings:

In the past it has been demonstrated that EAE can be induced by the injection of murine spinal cord in complete Freund's adjuvant followed by two boosts of B. pertussis given intravenously 1 and 3 days later. SJL, A.SW,

DBA/1, B10.S animals develop severe clinical disease with associated CNS pathology. A.TH and A.TL mice show mild diseases, primarily manifested by weight loss which is accompanied by low grade histological lesions. Balb/c and P/J are generally resistant but histological lesions are found in an occasional animal. No evidence of disease was seen in AKR and C57BL/10 animals.

An important variable in these studies was the use of pertussis. This markedly complicated investigations of the disease because considerable variations exists among the capacity of different strains of pertussis to produce disease. Consequently, other variables were systematically studied. These included the source and dose of spinal cord as well as the dose of mycobacteria. It was found that high doses of either mycobacteria or spinal cord suppress the disease. An optimal schedule that does not require the use of pertussis was developed for the production of the disease in SJL mice. Many of these animals developed recurrent disease. The neuropathological findings showed perivascular cuffing, a predominant polymorphonuclear response, and extravasation of fibrin and red cells. Primary demyelination was a transient, early feature of the disease process but nerve fiber depletion and gliosis occurred as the disease progressed. The observed myelin degradation most commonly involved the ingestion by macrophages of small fragments of dissociated myelin via crypts or infoldings of the cell surface at the bases of which were pinocytotic coated vesicles. A similar pattern of myelin breakdown has been described in mouse hepatitis virus encephalomyelitis and multiple sclerosis.

The formation of antibody to BP is related to a number of variables including the type of mycobacteria, the dose of mycobacteria, the use of pertussis and the strain of mouse. The relationship of this response to the genetic background, and specifically to the H-2 haplotype, was investigated. H-2<sup>k</sup> and H-2<sup>d</sup> animals were found to be high responders with all types of adjuvants; mice with H-2<sup>b,d,p,q,s</sup> haplotypes were poor responders, after primary immunization; however, some H-2<sup>s</sup> and H-2<sup>d</sup> animals showed an increase after boosting. The use of M. tuberculosis instead of M. butyricum resulted in greater antibody formation in H-2<sup>d</sup> strains. The response in four congenic pairs of mice support the association between antibody formation and the H-2 complex findings with recombinant strains indicate that responsible genes reside in the I-A region of the H-2 complex. These observations coupled with those on susceptibility indicate that production of antibody to BP and susceptibility to EAE may be dissociated and under the control of different genes.

EAE is believed to be cell-mediated. Studies of the T-cell response to BP in the mouse have been initiated. Efforts are currently under way to transfer the disease with lymphoid cells. In these experiments an approach which was developed in our laboratory some years ago is being employed. This involves placing lymphoid cells from immunized mice in tissue cultures with BP. After optimal incubation, 2-4 days, the cells are transferred into syngeneic recipients. Our preliminary findings indicate that the approach will be effective in SJL mice.

Significance to Biomedical Research and the Program of the Institute:

A major function of the immune response is to provide protection against infectious agents. Similar mechanisms can lead to disease through either autoimmunity or immunopathologic reactions. It is becoming increasingly apparent the control and regulation of the immune response is complex and varies greatly. In the present project, experimental animals which can be controlled in regards to age, sex and genetic background are used. Well-characterized antigens are being used to dissect various parameters of immune regulation which can lead to neurological disease. In addition to producing new information about pathogenesis of experimental disease, this project is providing background for the development of new approaches and techniques to study human diseases as outlined elsewhere (Project Z01 NS 02202-06 NI).

Proposed Course:

Future studies will focus on prevention and modification of EAE and investigation of immunoregulatory factors which influence the immunopathologic process. Our studies to date have surveyed a number of mouse strains; future investigations will focus on selected strains which are high responders, low responders and those which develop EAE. Extensive neuropathological evaluation of the chronic relapsing form of EAE will be constituted. Attempts to adoptively transfer murine EAE with lymphoid cells will be expanded. If the disease can be effectively and reproducibly transferred with immune cells these will be characterized using lymphocyte markers.

Publications:

Barnett, L.B. and McFarlin, D.E.: Genetic control of antibody production to myelin basic protein in mice. J. Neuroimmunol. 1: 53-59, 1981.

Brown, A. and McFarlin, D.E.: Relapsing EAE in the SJL/J mouse. Lab. Invest. (In press).



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SUMMARY OF WORK (200 words or less - underline keywords)  <p>The purpose of this study is to examine the <u>host immune response to viruses</u> which can produce either acute or chronic infections of the CNS. These studies will examine the host immune response and its relationship to mechanisms of protection as well as disease production. In addition, attention will be directed at the immune response to viruses in order to permit identification of <u>disease associated abnormalities</u>.</p>																																		

Project Description:

Objective: This project is designed to examine the virus-host interaction of various models of virus-induced central nervous system disease. These studies focus on the role of several variables thought to be important in disease production. These include the biological properties of the virus, the immune response of the host to the virus and the influence of the genetic background of the host on disease susceptibility. In particular, the roles of these variables in establishing chronic viral infections of the CNS, as well as in control or potentiation of disease are being examined. These investigations are being performed in the mouse since the immunogenetic parameters of the host are well defined and easily manipulated.

In addition this project includes studies of the normal immune response in man to viruses associated with neurological disease. These studies are designed to examine the various components of the normal cellular immune response to these viruses and to establish their functional significance.

Methods Employed:

## A. Virus-induced CNS disease

Acute virus-induced disease of the CNS is being studied in animals infected with either mouse adapted measles virus or vesicular stomatitis virus (VSV). In addition, these viral infections are being used to study the mechanisms by which the acute disease can be modified to produce a subacute or chronic infection.

1. Measles virus. The hamster neurotrophic strain of measles virus adopted to the mouse is being used in these studies. This virus produces an acute CNS disease when inoculated IC into susceptible mice. Mechanisms of persistence are being examined in strains of mice which are less susceptible. Both susceptible and nonsusceptible animals are being examined with respect to their ability to support virus replication, and their ability to respond to the virus immunologically. Further, the role of antibody in the establishment of persistent or chronic infection is being investigated. This includes studies of mouse hyperimmune serum as well as monoclonal antibodies to specific measles virus polypeptides.

2. VSV. Inoculation of animals with wild type VSV produces an acute disease of the CNS resulting in death in 2-3 days post inoculation. The mechanisms by which this acute infection can be modified are being examined in studies using the R<sub>1</sub> mutant of VSV. This mutant is not temperature sensitive and replicates readily both *in vitro* and *in vivo*. However, R<sub>1</sub> produces considerably less tissue damage than does WT VSV. This has been reported to be due to a mutation which eliminates the virus-induced shutdown of host cell protein, an event associated with virus-induced cell damage.

The neuropathological characteristics of the diseases produced by WT and R<sub>1</sub> viruses are being studied using routine histological technique, immunofluorescence, and electron microscopy. The nature of the immune response to these viruses is being carried out using techniques similar to those employed for measles virus. In addition, the growth curves are being studied in brain and spinal cord and the R<sub>1</sub> virus is being examined for reversion to WT in vivo.

#### B. Virus specific immune response

The cellular immune response to measles, mumps and vaccinia viruses is being studied in normal individuals. The reactivity of T cells and T cell subsets is being examined using a lymphoproliferative assay performed on virus infected monolayers. T cells are being separated into the T<sub>Y</sub> and T<sub>nonY</sub> subsets on the basis of their ability to bind the Fc portion of the IgG. Further, identification of subsets is being prepared using monoclonal antibodies to subpopulations of T cells.

#### Major Findings:

##### A. Measles virus

Studies of measles virus induced CNS disease in mice have focused on the ability of different host characteristics to alter disease. Inoculation of BALB/c mice with hamster neurotropic measles virus (HNT) produces an acute encephalopathy with death occurring 12-14 days after inoculation in 70-90% of the mice. In contrast, inoculation of SJL/J mice with HNT virus results in acute disease in only a small proportion of the animals [20-30%]. However, about one third of the survivors develop a chronic encephalitis many weeks after inoculation. This late disease was associated with a persistence viral antigen in the neurons throughout the brain, but predominantly in the limbic system. Clinically the late disease is characterized by wasting, seizures and focal paralysis culminating in death. Although minimal histological changes are found in the acute disease, the later disease in SJL mice is characterized by a marked inflammatory response. These studies demonstrate the ability of the same agent, HNT virus, to produce different disease patterns in mice with different genetic backgrounds. This model will permit analysis of genetic and immunological factors which allow for persistent or chronic infection with measles virus.

Similarly, the effect of modification of the host immune response on disease susceptibility has been studied using HNT infection in BALB/c mice. In these studies it has been shown that passive transfer of mouse serum with high levels of antibody to measles virus 3 days after inoculation of mice with HNT virus abolishes the acute disease. However, approximately 30% of these animals develop a subacute disease 30-40 days post inoculation. Similar to the pathological findings associated with the chronic disease in SJL mice, the late disease in animals passively immunized was characterized by an inflammatory reaction in the meninges and parenchyma of the brain.

Fluorescent antibody studies in these mice indicate that the appearance of viral antigen in the central nervous system is delayed until approximately 2 weeks post inoculation and then persists until the onset of clinical signs. Thus, these findings demonstrate the ability of elevated levels of antibody early in the infection to modify the normally acute disease and to produce a chronic infection which results in a late disease in a substantial proportion of animals.

The role of antibody in the production of a chronic infection has been more clearly defined using various monoclonal antibodies to measles virus polypeptides. Transfer one of the monoclonal antibodies to the HA of measles virus produces an effect similar to that of hyperimmune serum. In contrast, transfer of antibody directed at the nucleocapsid of measles virus had no demonstrable biological effect *in vivo*. These studies, therefore, will hopefully define more clearly the role of various components of the humoral immune response in chronic infections.

## 2. VSV

In infections with wild type (WT) VSV results in a rapidly fatal disease leading to death in 2-3 days. Fluorescent antibody studies have demonstrated viral antigen throughout the brain, but restricted to neurons. However, inoculation of mice with various temperature sensitive mutant strains have been shown to produce a more subacute form of disease manifested by hindlimb paralysis due to involvement of the anterior horn cells of the spinal cord. Thus, altered disease patterns have been attributed to the temperature sensitivity of mutants employed. This has been further studied using a non-ts mutant R<sub>1</sub> which, unlike other VSV variants does not stop host protein synthesis. Consequently, R<sub>1</sub> replicates efficiently, but produces delayed cytopathic effect *in vitro*. R<sub>1</sub> has also been demonstrated to be a potent interferon stimulator. In contrast to the acute encephalitis and death 2-3 days post infection with WT VSV, R<sub>1</sub> produces a paralytic disease 5-6 days post infection with death occurring 6-7 days post infection. The R<sub>1</sub> VSV infection is productive in contrast to infections with ts mutants. Morphological studies showed localization of virus replication primarily in the anterior horn cells, but unlike WT VSV infections there is sparing of the ependymal cells. In addition, morphological evidence of transynaptic spread of R<sub>1</sub> virus has been found. Although altered CNS disease resembles those produced by VSV temperature sensitive mutants, in these studies it was produced by a stable non-ts revertant. This virus is associated with an alteration in incubation time, cell susceptibility and, most importantly, in the clinical disease which it produces. Thus, these studies clearly demonstrate the ability of a specific alteration of the viral genome to modify the clinical disease produced by this virus.

## 3. Cellular immune response to viruses in man

The cellular immune response to measles, mumps and vaccinia viruses has been studied using a lymphoproliferative assay. Although a substantial



response of PBLs can be demonstrated to mumps and vaccinia viruses, only marginal proliferation can be demonstrated to measles virus. However, each of these viruses is associated with long term immunity and substantial levels of antibody to mumps and measles persist for many years after infection.

Separation of PBL into T or B cell populations indicates that the response to mumps and vaccinia viruses is predominantly in the T cell population, although a significant B cell response has been noted to mumps. Again, only marginal stimulation was obtained to measles with the purified T cell fraction. Experiments using T cells further separated into the T<sub>Y</sub> and T<sub>nonY</sub> fractions have indicated that the major responding cell to mumps and vaccinia viruses is in the T<sub>Y</sub> population, a population associated with suppression in other *in vitro* assays. In a few individuals found to be capable of responding to measles virus, this response is also restricted to the T<sub>Y</sub> fraction.

#### Significance to Biomedical Research and the Program of the Institute:

The importance of genetic and immunological factors in susceptibility, potentiation or protection in viral infections is not well established. Further, although the properties of the infectious agent are certainly of major importance in determining the effect of infection on the host, the actual virological characteristics important in producing persistent infection in distinction to cell death are largely unknown. The delineation of the normal cellular immune response to viruses associated with neurological disease in man will allow a more precise identification of abnormalities which may be related to specific disease states. It is hoped that the studies outlined in this project will help to identify the host and virological factors involved in the production of chronic neurological diseases, such as Subacute Sclerosing Panencephalitis (SSPE) and possibly Multiple Sclerosis (MS) and Amyotrophic Lateral Sclerosis (ALS).

#### Proposed Course:

These studies will focus on the various host mechanisms responsible for persistence of virus and modification of acute viral infections using the measles virus model. Major attention will be given to the role of immunological mechanisms in protection and disease production in this example of persistent viral infection. Further, studies will be directed at establishing the significance of the T<sub>Y</sub> restricted response in man and to establishing the functional roles of the T cell subpopulations in man.

#### Publications:

Greenstein, J.I., Baron, A.G.S., Lazzarini, R. and McFarland, H.F.: Infection of the central nervous system produced by R<sub>1</sub> vesicular stomatitis virus (R<sub>1</sub>-VSV). Lab. Invest. 44: 487-495, 1981.

Rammohan, K.W., McFarland, H.F. and McFarlin, D.E.: Induction of subacute murine measles encephalitis by monoclonal antibody to virus hemagglutinin. Nature 290: 588-599, 1981.









# ANNUAL REPORT

October 1, 1980 through September 30, 1981

Laboratory of Molecular Genetics  
National Institute of Neurological and Communicative Disorders and Stroke

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ANNUAL REPORT  
October 1, 1980 through September 30, 1981  
Laboratory of Molecular Genetics  
National Institute of Neurological and Communicative  
Disorders and Stroke

Robert A. Lazzarini, Acting Chief

The Laboratory of Molecular Genetics was officially established on January 30th of this year. The laboratory investigates the molecular events involved in the duplication and expression of the mammalian and viral chromosomes, and at present has two sections, a Molecular Virology Section and a Recombinant Genetics Section.

Thus far the major efforts have been administrative, largely directed toward recruiting personnel, acquiring designated space, and ordering equipment. A laboratory office has been established and staffed in temporary quarters. The present scientific staff is derived from the members of the former Molecular Virology Section (LMB, NINCDS), which was transferred to the new laboratory at its inception. The recruitment of additional scientific staff has been deferred until the hiring moratorium is lifted.

The progress of the laboratory programs reflect the staffing profile---a number of substantive advances have been made in the Molecular Virology program, while the Recombinant Genetics program has not yet commenced. However, the programs are closely interrelated, and many of the advances in the Virology Program, such as the construction of specialized vectors for the study of gene expression, will directly benefit the Recombinant Genetics program.

The advances made in the Molecular Virology program during the last year fall into four categories.

1. A generalized model for the origin of defective interfering (DI) particles. DI particles are small virus particles that arise naturally during the replication of most infectious virus. Unlike their infectious parents, the DI particles are innocuous to the host. When they happen to co-infect a cell together with infectious virus, they suppress (interfere) with the growth of the infectious virus. These naturally occurring particles have been shown to be very effective anti-viral agents. Our studies of the chromosomal structure of DI particles have demonstrated that there are at least four vastly different types of DI particles among the negative-strand RNA viruses. This year, the work has culminated in a proposal for the origin of the diverse types of DI particles. The proposal stipulates that aberrant replicative events, rather than recombinational events, are responsible for the generation of the DI particles. During these illegitimate replicative events, the RNA polymerase jumps from one template to another one in close proximity. The polymerase, however, does not release the daughter strand that it was synthesizing but continues to elongate it, copying the new template. Consequently, the finished daughter strand is a hybrid and contains information from two different templates. The four types of DI particles are thought to result from four specific scenarios of this generalized scheme in which the polymerase has made different or multiple transcriptional leaps.

Armed with the new insights into the origin of DI particles, it should be possible to design specific agents which will stimulate the type of aberrant replicative events

that give rise to DI particles. Since DI particles specifically interfere with viral infections, the stimulated production of them should have considerable therapeutic potential.

2. Pathogenicity of Mumps Virus modulated by DI particles. In some virus-host cell combinations, the proliferation of DI particles is so abundant that artificial stimulation is not necessary. The Enders' vaccine strain of Mumps virus and certain mammalian cells lines are such a combination. This combination gives rise to a low level, though persistent infection which exhibits minimal pathology and is self-limiting after many cell passages. Our biological and biochemical studies indicate that defective interfering particles are generated very early in the infection and that they are replicated throughout the course of the persistent infection. These infections eventually cure themselves when the DI particles become so abundant that they prevent further replication of the infectious virus. In contrast, the virulent (wild type) strain of Mumps virus gives rise to an acute infection in the same cells and causes a massive pathology ultimately leading to cell death. Thus, the critical property of the vaccine virus--minimal pathogenicity in mammalian cells--may be related to the abundant production of DI particles. The possibility that the ability to spawn DI particles is closely related to the attenuation observed in other viral vaccines is being investigated.

3. Recombinant DNA cloning of VSV sequences. Previous studies from our laboratory indicate that the terminal regions of the VSV chromosome are central in the regulation of viral replication. However, final verification of this proposition depends upon separating these terminal sequences from the rest of the viral genome and manipulating the sequences at will. Currently the only means of doing this is through the use of recombinant DNA techniques. During the last year, we have successfully synthesized DNA copies of the terminal regions of the VSV chromosome and have cloned these materials in *E. coli* using a plasmid vector. We have completed the cloning phase of our work and now have all of the clones we will need. This is the first step in a long-term program in which the individual regulatory regions controlling the attachment of the RNA polymerase to the chromosome, the initiation of RNA synthesis and the assembly of the newly synthesized RNA into a nucleocapsid will be identified and manipulated.

4. The expression of viral proteins in chimeric cells. In order to dissect the growth cycle of viruses and to study the interaction of the various viral and host components, it is often necessary to isolate the component processes. With this strategy in mind we have undertaken a study of the assembly of nucleocapsid protein to the viral chromosomal RNA--a process central to the growth of all negative-strand RNA viruses. To isolate the process we will produce the nucleocapsid protein in chimeric cells which contain a cloned copy of the viral nucleocapsid gene, appropriately positioned in a suitable vector so that it will be expressed. During the last year we have obtained twenty clones of portions of the nucleocapsid protein gene. The complete gene will be constructed by joining portions of two clones that together cover the entire gene.

We have completed the construction of a vector to carry the assembled nucleocapsid gene into the host cell. This complex vector, which contains both SV<sub>40</sub> and bacterial plasmid sequences, will express genes inserted into it as if they were authentic SV<sub>40</sub> genes. The bacterial plasmid sequences enable us to grow the SV<sub>40</sub>--VSV recombinant DNA in *E. coli* and to obtain large amounts of it. Although this vector was specifically designed for use with the nucleocapsid gene, it is ideally suited for the study and expression of almost any mammalian gene.

CONTRACT NARRATIVE  
Laboratory of Molecular Genetics  
Fiscal Year 1981

UNIVERSITY OF VIRGINIA (NOI-NS-12353)

Title: Large Scale Preparation of VSV and its DI Particles

Contractor's Project Director: Dr. Jay C. Brown

Current Annual Level: \$80,000

Objectives: To establish conditions for the growth and purification of VSV and four of its defective particles which will reproducibly yield materials of the requisite purity and activity, and to supply such materials to the Laboratory of Molecular Genetics, IRP/NINCDS.

Major Findings:

a) Conditions and procedures have been devised for the purification of the virus particles. Materials prepared by this new scheme meet the specifications set forth in the contract.

b) The contractor has delivered to the Laboratory of Molecular Genetics, IRP/NINCDS, the amounts of purified VSV and DI particles stipulated in the contract.

Significance to the NINCDS Program and Biomedical Research: The procedures and materials developed under this contract are immediately used by the Molecular Genetics Laboratory. This contract, therefore, forms an integral part of the Laboratory's research program, namely, the regulation of viral nucleic acid synthesis in animal cells. This contract has supplied the Program with the raw materials for RNA sequencing of the viral genomes. These studies have characterized sites on the chromosomes that are important for autointerference, DI particle genesis and the replication of the viral genome.

SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  <div style="text-align: center; font-weight: bold;">Z01 NS 02026-09 LMG</div>																														
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PI: R. A. Lazzarini, Acting Chief, Lab. of Molecular Genetics    LMG    NINCDS																																
OTHER: <table style="width: 100%; border: none;"> <tr> <td style="width: 35%;">M. Schubert</td> <td style="width: 35%;">Staff Fellow</td> <td style="width: 30%;">LMG NINCDS</td> </tr> <tr> <td>J. Condra</td> <td>Staff Fellow</td> <td>LMG NINCDS</td> </tr> <tr> <td>M. McCarthy</td> <td>IPA</td> <td>LMG NINCDS</td> </tr> <tr> <td>F. Yang</td> <td>Visiting Fellow</td> <td>LMG NINCDS</td> </tr> <tr> <td>Y. Kang</td> <td>IPA</td> <td>LMG NINCDS</td> </tr> <tr> <td>S. Yamaguchi</td> <td>Psychologist</td> <td>LMG NINCDS</td> </tr> <tr> <td>J. Sprague</td> <td>Chemist</td> <td>LMG NINCDS</td> </tr> <tr> <td>H. Arnheiter</td> <td>Guest Worker</td> <td>LMG NINCDS</td> </tr> <tr> <td>I. Chien</td> <td>Chemist</td> <td>LMG NINCDS</td> </tr> <tr> <td>G. Harmison</td> <td>Chemist</td> <td>LMG NINCDS</td> </tr> </table>			M. Schubert	Staff Fellow	LMG NINCDS	J. Condra	Staff Fellow	LMG NINCDS	M. McCarthy	IPA	LMG NINCDS	F. Yang	Visiting Fellow	LMG NINCDS	Y. Kang	IPA	LMG NINCDS	S. Yamaguchi	Psychologist	LMG NINCDS	J. Sprague	Chemist	LMG NINCDS	H. Arnheiter	Guest Worker	LMG NINCDS	I. Chien	Chemist	LMG NINCDS	G. Harmison	Chemist	LMG NINCDS
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COOPERATING UNITS (if any) Department of Neurology, Laboratory of Neurovirology, Johns Hopkins University, School of Medicine; Department of Microbiology, Duke University School of Medicine, Division of Virology, University of Cambridge																																
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<p>The long range objective of this project is the description of the component molecular events involved in the replication of the negative strand viruses (myxo, paramyxo, rabdo, arena and bunya viruses). The topics that are currently being investigated are:</p> <ol style="list-style-type: none"> <li>1. The origin of DI particles.</li> <li>2. The mechanism of mRNA synthesis in VSV infected cells.</li> <li>3. Nucleocapsid assembly.</li> </ol> <p>This project was transferred from the Laboratory of Molecular Biology.</p>																																



## Project Description:

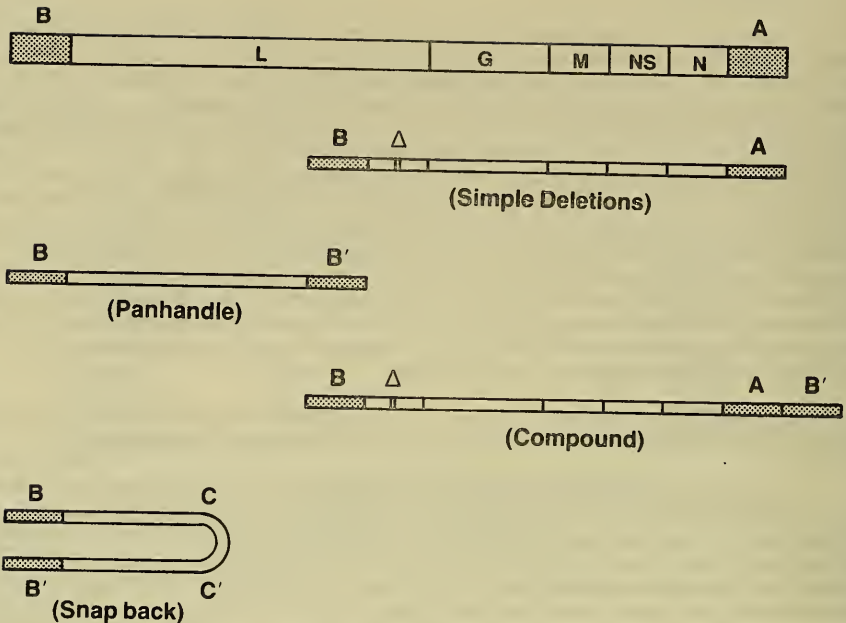
**Objectives:** Viral diseases of the central nervous system (CNS) usually occur as a complication rather than a normal consequence of infection. Nevertheless, many members of the myxo-, paramyxo-, and rhabdovirus families, either exceptionally or as a normal consequence, infect the CNS, causing encephalitis or meningitis. Despite their importance to medical neurology, very little is known about the regulation and mode of replication of these viruses in the host organism. From what little is known, it is clear that their replication is very different from that described for polio virus or the RNA tumor viruses. Furthermore, the myxo-, paramyxo-, rhabdovirus infections also are distinguished in that they frequently elaborate defective interfering (DI) particles and exhibit evidence of autointerference and viral persistency. The description of the component molecular events involved in the replication of these viruses; the generation of defective interfering particles, autointerference and viral persistency are the subject of this project. It is anticipated that the study will delineate characteristics that can be exploited in containing and limiting viral infection to non-neural tissues or in the treatment or prevention of the viral infection.

**Major Findings:** Four major areas of our program have been pursued during the last year, and although interrelated, each area will be discussed separately.

1. The genesis of defective interfering particles. Over the course of the last several years, we have characterized the viral chromosomes of defective interfering (DI) particles in the belief that such studies would ultimately allow us to deduce the mechanism by which these particles are formed. These studies have employed electron-microscopy, molecular hybridization and RNA sequence analysis. The results have demonstrated that there are at least four general types of DI particles. In the figure, the upper bar is a schematic representation of the parental virus chromosome with its five genes (L, G, M, NS, and N) and the terminal sequences, A and B. The chromosomes from the four types of DI particles are illustrated below it. The SIMPLE DELETION has lost most of the L gene but retains the parental terminal sequences A and B. In contrast the PANHANDLE DI has lost most of the right hand portion of the parental genome and has complementary terminal sequences B and B'. Another type of DI is called "SNAP-BACK" because it is self-complementary over its entire length and when deproteinized snaps into a hair-pin duplex structure. The four and most complex type of DI, the COMPOUND DI is probably derived from the deletion DI by a second aberration during replication. It has a compound 3' terminus containing both the A and B' sequence.

Our studies have culminated during the last year, in the formal presentation of a generalized model for the origin of defective interfering particles. This model stipulates that aberrant replicative events rather than cleavage and reunion are responsible for the generation of DI particles. During these illegitimate events, the RNA polymerase jumps from one template to another in close proximity. The polymerase, however, does not release the daughter strand that it is synthesizing but continues to elongate it, copying the new template. Consequently, the finished daughter strand is a hybrid and contains information from two different templates. This strand is the progenitor of DI chromosomes. The four types of DI particles are seen to result from four specific scenarios of this generalized scheme in which the polymerase has made different or





multiple transcriptional leaps. Thus, the ability to generate DI particles is an intrinsic property of the polymerase that is enhanced or diminished depending upon the host-cell infected. We believe that this is an important characteristic of the negative strand RNA viral polymerases and one which can be exploited in developing new therapeutic modalities for viral diseases.

2. Pathogenicity of Mumps virus modulated by DI particles. Paramyxoviruses manifest diverse host-virus interactions ranging from acute, highly cytopathic infections to persistent non-cytopathic infections. The nature of the interaction depends on both host-cell and infecting virus. A major strategy in the development of viral vaccines--viral strands that exhibit minimal cytopathology--has been adaptation of the virus to a different host to alter the host-virus interactions. For Mumps virus this has involved adaptation of human isolates to chick embryo.

We have demonstrated that the Enders' vaccine strand of Mumps virus infects mammalian cells giving rise to a lower level, persistent infection which exhibits minimal pathology and is self-limiting. Our biochemical and biological studies indicate that defective interfering particles are generated early in the infection and that they

are replicated throughout the course of the persistent infection. The population of DI RNAs changes throughout the course of the persistent infection and become more heterogeneous with each cell passage. Many of these RNAs exhibited "snap-back" character similar to that observed in well characterized DI particles of VSV. The infection eventually cures itself when the DI particles become so abundant that they prevent further replication of the infectious virus. This selective amplification is specific to the host-virus interaction since the same stock of Enders' virus does not yield detectable amounts of DI RNA when grown in chick embryo. Furthermore, the virulent (wild-type) strain of Mumps gives rise to an acute infection in the same cells causing a massive pathology that ultimately leads to cell death. Thus the critical property of the vaccine virus--minimal pathogenicity in mammalian cells--may be related to the abundant production of DI particles. The possibility that the ability to spawn DIs is closely related to the attenuation observed in other viral vaccines is being investigated.

3. **Recombinant DNA Cloning of VSV Sequences.** The genome of all negative strand RNA viruses is a single strand of negative polarity RNA. Replication of the virus proceeds through the synthesis of a full-length complementary (positive) strand which is then used as a template for synthesis of progeny negative strands. All full-length RNAs of either polarity exist as RNase-resistant nucleocapsids, in tight association with the viral N (nucleocapsid) protein.

Several lines of evidence suggest that the balance between mRNA transcription and genome replication may be controlled, at least in part, by nucleocapsid assembly, and that initiation of encapsidation may occur at a specific site near the 5' end of the viral RNA strands. A direct demonstration of a nucleation site has been hampered, however, by the inability to prepare viral N protein in a soluble, active form. So far, cell-free systems have not been shown to carry out the earliest stages of nucleocapsid assembly, so we have chosen to study this process in VSV-infected cells.

We have constructed DNA copies of the genome extending from the precise 3' terminus. These cDNAs have been cloned into bacterial plasmids and M13 phages and partially sequenced. We have also constructed an SV40 cloning vector suitable for expression of these sequences in monkey cells. This can be grown as a bacterial plasmid and carries a large deletion in the major SV40 capsid protein (VP1) gene, but retains the normal splicing and polyadenylation sites for the mRNA. We have replaced the deleted material with the 3'-VSV cDNA, allowing synthesis of an SV40-VSV hybrid transcript containing the putative nucleation site for the N protein.

Co-infection of monkey cells with this recombinant and VSV helper (to provide N protein) should lead to encapsidation of the hybrid SV40-VSV transcript directed by its N protein binding site. After the cDNA fragment carrying the nucleation site has been identified, its precise location will be determined by localized mutagenesis of the cloned cDNA.

Since an N protein nucleation site must also exist near the 5' end of the negative strand, a similar approach will be taken for its identification using the clones of the 5' end that we have constructed.

Since binding of the viral RNA polymerase to its template occurs on nucleocapsids rather than on naked RNA transcripts, this should also permit us to study the sequences involved in polymerase recognition and other components of the replication apparatus.

4. Expression of Cloned Viral Genes in Eukaryotic Cells. In order to define the biological roles of the various viral proteins, it is desirable to study them in an intracellular environment. To accomplish this, we are engaged in cloning viral genes to study their expression in mammalian cells.

Our first objective is to construct a cDNA clone of the complete gene for the nucleocapsid protein, using two overlapping cDNA fragments that together represent the entire gene. We are presently in the process of joining these fragments. The final product will then be cloned into the SV40 expression vector described above.

Proposed Course of Project: 1. To further explore the details of viral replication and transcription with hybridoma cell lines that produce antibodies against specific viral proteins and to using these to dissect viral processes.

2. To investigate the biochemical events leading to the assembly of nucleocapsids from precursor proteins and RNA. Efforts will be made to investigate the regulation and orchestration of this process with recombinant DNA techniques.

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ANNUAL REPORT

October 1, 1980 through September 30, 1981

Laboratory of Experimental Neurology

National Institute of Neurological and Communicative Disorders and Stroke

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PROJECT REPORTS

Anatomical and Functional Sequelae of Penetrating Head Injury	1
Z01 NS 02189-06 LEN	
Anatomical Corollary for Computed Tomography	2
Z01 NS 02428-02 LEN	



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE NOTICE OF INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02189-06 LEN
PERIOD COVERED October 1, 1980 through September 30, 1981		
TITLE OF PROJECT (80 characters or less) Anatomical and Functional Sequelae of Penetrating Head Injury		
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT		
PI:	W.F. Caveness	Chief LEN, IRP, NINCDS
OTHER:	T.M. Boehm	Chief, Clinical Investigation Service, WRAMC (Walter Reed Army Medical Center)
	E.D. George	Chief, Neurosurgical Service, WRAMC
	C.B. Early	Chief, Neurosurgical Service, NMMC (National Naval Medical Center)
COOPERATING UNITS (if any) Walter Reed Army Medical Center, Washington, D.C. National Naval Medical Center, Bethesda, Maryland Research Service, Veterans Administration, Washington, D.C.		
LAB/BRANCH Laboratory of Experimental Neurology		
SECTION		
INSTITUTE AND LOCATION NINCDS, NIH, Bethesda, Maryland 20205		
TOTAL MANYEARS: 0.50	PROFESSIONAL: 0.33	OTHER: 0.17
CHECK APPROPRIATE BOX(ES) <input checked="" type="checkbox"/> (a) HUMAN SUBJECTS <input type="checkbox"/> (b) HUMAN TISSUES <input type="checkbox"/> (c) NEITHER  <input type="checkbox"/> (a1) MINORS <input type="checkbox"/> (a2) INTERVIEWS		
SUMMARY OF WORK (200 words or less - underline keywords) The objective of this study is to determine the loss in brain substance and the alteration in brain function twelve (12) years after brain damage incurred during the Vietnam War. The resource for this project is a Registry of 1,500 head injuries compiled in the field by military surgeons from 1967 to 1970, and a subsequent Records Review carried out between 1975 and 1979. This study will be accomplished by an in-hospital examination of 1,000 brain-damaged subjects and 200 controls, the latter being utilized as an unique approach to the loss or alteration in structure, i.e., computerized axial tomography and other techniques not available in previous studies of functional sequelae.  This project has been terminated at NINCDS, NIH, because of the death of the principal investigator; however, it continues currently at the Walter Reed Army Medical Center under the direction of Lt.Col. Daniel Dillon, Neurosurgeon, USAR, in the Department of Clinical Investigation Service. Another LEN study, "Anatomical Corollary for Computed Tomography" (Z01 NS 02428-02, which please see) has been incorporated into this larger study.		



SMITHSONIAN SCIENCE INFORMATION EXCHANGE PROJECT NUMBER (Do NOT use this space)	U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES PUBLIC HEALTH SERVICE <b>NOTICE OF</b> INTRAMURAL RESEARCH PROJECT	PROJECT NUMBER  Z01 NS 02428-02 LEN												
PERIOD COVERED <p style="text-align: center;">October 1, 1980 through September 30, 1981</p>														
TITLE OF PROJECT (80 characters or less)  <p style="text-align: center;">Anatomical Corollary for Computed Tomography</p>														
NAMES, LABORATORY AND INSTITUTE AFFILIATIONS, AND TITLES OF PRINCIPAL INVESTIGATORS AND ALL OTHER PROFESSIONAL PERSONNEL ENGAGED ON THE PROJECT  <table style="width: 100%; border: none;"> <tr> <td style="width: 33%;">PI:</td> <td style="width: 33%;">W.F. Caveness</td> <td style="width: 33%;">Chief</td> <td style="width: 33%;">LEN, IRP, NINCDS</td> </tr> <tr> <td>OTHER:</td> <td>M.B. Carpenter</td> <td>Chairman, Dept. of Anatomy</td> <td>USUHS</td> </tr> <tr> <td></td> <td>J. Hanaway</td> <td>Anatomist, St. Louis, MO</td> <td></td> </tr> </table>			PI:	W.F. Caveness	Chief	LEN, IRP, NINCDS	OTHER:	M.B. Carpenter	Chairman, Dept. of Anatomy	USUHS		J. Hanaway	Anatomist, St. Louis, MO	
PI:	W.F. Caveness	Chief	LEN, IRP, NINCDS											
OTHER:	M.B. Carpenter	Chairman, Dept. of Anatomy	USUHS											
	J. Hanaway	Anatomist, St. Louis, MO												
COOPERATING UNITS (if any) <p style="text-align: center;">Uniformed Services University of the Health Sciences (USUHS), Dept. of Anatomy, Bethesda, Maryland</p>														
LAB/BRANCH <p style="text-align: center;">Laboratory of Experimental Neurology</p>														
SECTION 														
INSTITUTE AND LOCATION <p style="text-align: center;">NINCDS, NIH, Bethesda, Maryland 20205</p>														
TOTAL MANYEARS: <p style="text-align: center;">0.42</p>	PROFESSIONAL: <p style="text-align: center;">0.34</p>	OTHER: <p style="text-align: center;">0.08</p>												
CHECK APPROPRIATE BOX(ES) <div style="display: flex; justify-content: space-between; align-items: flex-start;"> <div> <input type="checkbox"/> (a) HUMAN SUBJECTS         </div> <div> <input checked="" type="checkbox"/> (b) HUMAN TISSUES         </div> <div> <input type="checkbox"/> (c) NEITHER         </div> </div> <div style="display: flex; justify-content: space-between; align-items: flex-start; margin-top: 5px;"> <div> <input type="checkbox"/> (a1) MINORS         </div> <div> <input type="checkbox"/> (a2) INTERVIEWS         </div> </div>														
SUMMARY OF WORK (200 words or less - underline keywords) <p>The objective of this study is to provide templates of anatomical structures from the human head at intervals of 1 mm from base to vertex. The horizontal sections will be parallel with a plane passing through the glabella and external auditory meatus. These will be used in interpreting the anatomy represented in CT scans obtained in the same horizontal plane, at 5 mm intervals, from base to vertex. To accomplish this, an LKB 2250 PMV Cryo-microtome is being modified to accommodate the human head. This instrument will be installed at the USUHS where the study will be conducted in collaboration with the Department of Anatomy. The product of this endeavor is primarily intended to enhance the interpretation of CT scans. A secondary objective is to provide a definitive ATLAS for universal use.</p> <p>This project has been terminated at NINCDS, NIH, because of the death of the principal investigator; however, it continues currently at the Walter Reed Army Medical Center, where it has been combined with: Anatomical &amp; Functional Sequelae of Penetrating Head Injury, Z01 NS 02189-06 LEN (which please see).</p>														











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